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EDITORIAL OFFICE
Jin-Lei Wang, Director

Xiu-Xia Song, Vice Director
World Journal of Gastrointestinal Oncology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
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TOPIC HIGHLIGHT

2016 Colorectal Cancer: Global view

MicroRNA in rectal cancer

Azadeh Azizian, Jens Gruber, B Michael Ghadimi, Jochen Gaedcke

Azadeh Azizian, B Michael Ghadimi, Jochen Gaedcke, Department of General, Visceral and Pediatric Surgery, University Medical Center Göttingen, 37075 Göttingen, Germany

Jens Gruber, Junior Research Group Medical RNA Biology, German Primate Center, 37077 Göttingen, Germany

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Correspondence to: Jochen Gaedcke, MD, Department of General, Visceral and Pediatric Surgery, University Medical Center Göttingen, Robert-Koch-Straße 40, 37075 Göttingen, Germany. jochen.gaedcke@med.uni-goettingen.de
Telephone: +49-551-3920933
Fax: +49-551-3912550

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Abstract

In rectal cancer, one of the most common cancers worldwide, the proper staging of the disease determines the subsequent therapy. For those with locally advanced rectal cancer, a neoadjuvant chemoradiotherapy (CRT) is recommended before any surgery. However, response to CRT ranges from complete response (responders) to complete resistance (non-responders). To date we are not able to separate in advance the first group from the second, due to the absence of a valid biomarker. Therefore all patients receive the same therapy regardless of whether they reap benefits. On the other hand almost all patients receive a surgical resection after the CRT, although a watch-and-wait procedure or an endoscopic resection might be sufficient for those who responded well to the CRT. Being highly conserved regulators of gene expression, microRNAs (miRNAs) seem to be promising candidates for biomarkers. Many studies have been analyzing the miRNAs expressed in rectal cancer tissue to determine a specific miRNA profile for the ailment. Unfortunately, there is only a small overlap of identified miRNAs between different studies, posing the question as to whether different methods or differences in tissue storage may contribute to that fact or if the results simply are not reproducible, due to unknown factors with undetected influences on miRNA expression. Other studies sought to find miRNAs which correlate to clinical parameters (tumor grade, nodal stage, metastasis, survival) and therapy response. Although several miRNAs seem to have an impact on the response to CRT or might predict nodal stage, there is still only little overlap between different studies. We here aimed to summarize the current literature on rectal cancer and miRNA expression with respect to the different relevant clinical parameters.

Key words: Polymorphism; MicroRNA; Rectal cancer; Response; Chemoradiotherapy; Expression

Core tip: In rectal cancer, a proper staging of the disease determines the subsequent therapy. Also, prediction of prognosis or therapy response could serve to individualize therapy. MicroRNAs (miRNAs) are highly conserved

regulators of gene expression, and seem to be promising candidates for biomarkers. Several miRNAs are part of a specific expression profile in rectal cancer tissue, while others have been correlated to clinical parameters and therapy response. However the comparison of different studies shows only little overlap and even partly oppositional results. Differences between analytical methods and tissue storage types can contribute to that. Further functional analyses are needed to fully understand the impact of miRNAs in rectal cancer.

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INTRODUCTION

Colon and rectal cancer

Taken together, colon and rectal cancer is the third most common cancer worldwide, accounting for 1.36 million newly diagnosed colorectal cancers in 2012[1] with rectal cancer accounting for 30%. The main purposes to differentiate between colon and rectal cancer are anatomical[2] and molecular differences[3]. Several studies have also shown that disease-correlated genetic and lifestyle factors differ between colon and rectal cancer[3-8]. Differences in survival, fewer inherited syndromes, and younger age at diagnosis in rectal cancer patients further strengthen the rationality of separating the two diseases[8].

Specifically due to the anatomical differences in comparison to colon cancer, local recurrence is a considerable concern in the treatment of rectal cancer. This led to the introduction of radiation in the treatment of rectal cancer patients and represents fundamental therapeutic differences to colon cancer. While treatment of upper rectal cancer provides primary surgical resection and can therefore be compared to colon cancer, the standard treatment of locally advanced cancer in the lower and middle rectum includes preoperative chemoradiotherapy (CRT), followed by total mesorectal excision (TME)[9]. The introduction of preoperative chemoradiotherapy requires additional challenges in diagnostics and therapy planning which differ from colon cancer, requiring a precise pretherapeutic staging.

The rectal cancer staging can be made according to the TNM staging system from the World Health Organization: The Union for International Cancer Control (UICC). Depending on tumor status, nodal status, and metastases, rectal cancer is subdivided in UICC Ⅰ-Ⅳ. While tumor status is determined by magnetic resonance imaging and trans-rectal endoscopic ultrasound, metastasis status is assessed by computed tomography of thorax and abdomen and ultrasound of the latter. Defining nodal status remains the most challenging and is evaluated today using all aforementioned imaging techniques. Correct staging of patients with rectal cancer is actually required at two time points: First, before starting any treatment, and second, after neoadjuvant chemoradiotherapy, because response to CRT is heterogeneous; it ranges from resistance to complete pathological response. Response to CRT, measured as tumor regression grade (TRG), correlates significantly with disease-free- and overall-survival. The first staging is crucial for deciding if a preoperative CRT is needed, hence only locally advanced stages receive CRT. The second staging acquires more and more importance with regard to the possibility of organ-preserving strategies, which have been recently suggested as an alternative to TME for patients that responded very well to CRT. Basis for this upcoming approach can be found in the side effects of rectal cancer surgery. However, if lymph node metastases are undetected these have to be considered as origin of local relapse. In this respect molecular markers may play an increasing role as potential predictive marker.

miRNAs

MicroRNAs (miRNAs) are short non-coding RNAs, 20-22 nucleotides in length discovered in 2001[10,11]. They are highly conserved between vertebrates, invertebrates and plants[12]. Through base-pairing with their target mRNA, miRNAs induce post-transcriptional gene silencing by mRNA degradation or translational blocking[13,14]. Consequently, they present master regulators of gene expression and therefore influence many physiological and patho-physiological processes[15].

Some conservatively estimated 60% of all human mRNAs are regulated by miRNAs, which represent virtually all cellular and molecular functions. Thus, it is not surprising that miRNAs are involved in diverse processes including embryonic development, cell differentiation, cellular proliferation, metabolism, adaptation to environmental stress, and apoptosis[13]. Thus, miRNAs play important roles in many human diseases, and even in the human aging process[16]. By now the impact of specific miRNAs is reported not only for almost every cancer type but also for other diseases like diabetes, cardiovascular diseases, neurological diseases and even psychological diseases like schizophrenic disorder. Therefore miRNAs are of great interest as possible biomarkers in various diseases due to their abundance and cell-type specificity.

Many human miRNA loci are located within intronic (miRtrons) regions[17,18]. While it is a general belief that intronic miRNAs are released from excised introns after the splicing, an interesting study of Kim *et al*[19] indicates that intronic miRNAs can be processed from unspliced intronic regions, ensuring both miRNA biogenesis and protein synthesis from a single primary transcript, supporting the assumption that the intronic miRNAs and their hosting genes are co-regulated[20]. miRNAs are transcribed by RNA Polymerase Ⅱ (pol Ⅱ). The primary transcripts (pri-miRNA) are 5′-capped and polyadenylated. They have at least one stem-loop structure that encodes an individual miRNA sequence within the stem. Drosha, a nuclear RNase Ⅲ type enzyme, and DGCR8, a double-

stranded RNA-binding protein, work as a complex known as microprocessor, which cleaves the primary structure of the pri-miRNA in a process called "cropping"[10]. The products of this reaction are the pre-miRNs, which are exported to the cytoplasm by exportin-5[21,22]. In the cytoplasm the pre-miRNAs are further processed by the cytoplasmic RNase Ⅲ called Dicer. Only one strand of the produced duplex of RNA is incorporated into the effector complex RNA-induced silencing complex (RISC), acting as the guide strand, while the passenger strand is rapidly degraded. However, either arm of the pre-miRNA can be selected to become the guide strand. The strand-selection differentially or coherently processes mature miRNAs, giving rise of gene regulatory RNAs with distinct target-spectra. This process of flexible arm selection has been reported for many small RNAs, including canonical and intronic miRNAs[23,24]. Eventually, RISC migrates to P-bodies to scan and bind to the 3′ untranslated region of the target mRNA.

The miRNA-mRNA binding is specific due to the sequence complementarity of the "seed" region of the miRNA. The canonical seed is a tract of 7-8 nucleotides usually located at the 5′ end of the miRNA molecule, which is fully base pairing one or multiple sites within the sequence of a target mRNA[25] capable of follow structures of miRNA-5"-seeds or alternative seed architectures.

Hence, the core of the target-region with high complementarity is short, which results in multitude of transcripts with possible binding sites for a given miRNA. Therefore a single miRNA has the potential to regulate hundreds of different mRNA targets[26], while on the other hand a single mRNA is regulated by diverse miRNAs simultaneously.

Genome-wide miRNA-expression-profiling studies have demonstrated a specific profile of upregulated and downregulated miRNAs in almost all cancer types[27,28]. In particular, due to their lack of complex post-transcriptional modifications in contrast to mRNAs and other RNA classes (rRNA, tRNA), the potential of miRNAs as biomarkers for cancer diagnosis, prognosis, and response to treatment is expected high. Not only miRNAs can be found in serum or plasma of patients and healthy individuals, but also in other body fluids such as tears, breast milk, bronchial lavage, colostrum and seminal, amniotic cerebro-spinal, pleural and peritoneal fluids[29]. The diagnostic potential of miRNAs relies in part on their stability to storage handling: miRNAs remain stable even in conditions most RNAs would normally degrade (extreme pH-levels, boiling, *etc.*)[30].

Cell-free and circulating miRNAs can be vesicle associated (exosomes and microvesicles), or stable AGO-miRNA complexes and became well accepted biomarkers for non-invasive biomarkers for numerous cancer types[31-36].

LITERATURE SEARCH

A systematic literature search was conducted using PubMed for "rectal cancer", "miRNA" and "miRNA". A total of 27 studies containing miRNA research from rectal cancer tissue, normal mucosa tissue, and body fluids were included for review. Five studies were involved in differential expression of miRNAs in rectal cancer, six studies explored specific miRNAs which showed a correlation to clinical parameters, and nine studies analyzed miRNAs concerning alteration during chemoradiotherapy and response prediction. Five studies conducted further *in vitro* analyses for rectal cancer specific miRNAs. Three studies were found to be dealing with polymorphism in miRNAs in rectal cancer patients.

DIFFERENTIAL EXPRESSION OF MIRNA IN RECTAL CANCER

It is widely accepted that tumors share specific oncogenic pathways. *Vice versa* the tissue of origin has also an impact on the molecular features of each tumor. These are of great interest as they may explain cellular processes such as carcinogenesis, progression, or therapy resistance. Accordingly, rectal cancer specimens and normal mucosa tissue were analyzed. In a first analysis Slattery *et al*[8] compared colorectal cancer tissue [formalin-fixed paraffin embedded (FFPE)] to normal tissue samples and also compared normal rectal tissue to normal colon tissue using microarray analysis. All samples were further subdivided according to their CpG island methylator phenotype status or the mutational status of *KRAS* or p53, revealing 129, 143, and 136 unique miRNAs respectively. The availability of miRNA expression data of normal colon and rectal tissue samples enabled a comprehensive comparison, which identified 73 differentially expressed genes (based on a two-fold fold change) and thus highlighted also important molecular differences between colon and rectal cancer. A comparable study by Li *et al*[37] involving miRCURY Array LNA miRNA chips and technical validation by RT-PCR analyzed expression profiles from six rectal cancer tissues and paired adjacent non-tumor tissue, which identified 67 upregulated and 39 downregulated miRNAs associated with rectal cancer. The number of rectal cancer tissues used (n = 6) is extremely low and, by using an array platform with several hundreds of miRNAs, it is required to correct for multiple testing. This did not occur; therefore the findings are potentially inapplicable.

In a larger study, our own group[38] used LNA-enhanced miRCURY microarrays to map the expression of 2090 miRNAs. Tumor biopsies and matched mucosa samples of 57 patients with locally advanced rectal cancer were profiled. Forty-nine miRNAs differed with high significance between normal and rectal cancer tissue, 20 of these 49 miRNAs were upregulated while 29 were downregulated in rectal cancer *vs* mucosa. Upon employing a combination of fold-change and *P*-value for selection, the expression of 10 miRNAs was validated using 48 samples (24 matched tumor-mucosa samples) by semi-qRT-PCR; in 8 of the 10 miRNA expression levels correlated very well with miRCURY data as they showed the same alteration in both methods and both sets of tissue.

Studies by Wang *et al*[39] could confirm that the expression level of two miRNAs (miR-34a, miR-200c) that were previously found to be differentially regulated in various types of cancer, also were significantly upregulated in rectal cancer by analyzing 72 rectal cancer samples *via* qPCR.

Comparison of different studies to identify overlapping miRNA expression differences, *e.g.*, between rectal cancer and normal tissue is subject of certain restriction: Starting from tissue retrieval (*e.g.*, taking the biopsy during rectoscopy *vs* tissue excision from the resected surgical specimen that obviously already has a certain ischemia time) over tissue storage (*e.g.*, liquid nitrogen, RNA later, formalin fixation) and tissue work up to the final application of the various techniques that are available for miRNA measurement (*e.g.*, miRNA arrays from different companies, qPCR, sequencing). In this specific application, the reference tissue is of importance. Biases arise depending on whether paired normal mucosa or mucosa from different patients were used as a reference. On the other hand, miRNAs that finally overlap between different studies attract attention as they may be represent basic differences between the compared tissues. In this respect we aimed to identify the overlap between published data sets that were previously introduced, which currently involve only two relevant datasets comparing rectal cancer and normal tissue[37,38]. Of these, 11 miRNAs were overlapping. Seven miRNAs were significantly upregulated (miRNAs 17, -18a, -21, -31, -135b, -223 and -492) while four were significantly downregulated (miRNAs-29c, -145, 147b and -375). In both studies also the expression of let-7f, miR-148 and -190 were significantly altered in rectal cancer, however they showed an oppositional regulation of these miRNAs comparing with the first two studies questioning their relevance for assessing differentially expression. For miR-145 even a third study performed by Wang *et al*[40] confirmed a significant in rectal cancer. Figure 1 shows an overview about the differential expression of miRNAs found according to the mentioned studies.

A closer look to the differentially expressed miRNAs reveals a broad range of different function. As a member of the miR-17/92 cluster miR-17 and 18a are both known to be involved in a large number of processes including normal development, tumorigenesis, immune-, cardiovascular-, and neurodegenerative diseases as well as aging[41]. Renal fibrosis[42], myelodysplastic syndroms[43], inflammatory processes[44], and especially cancer are only a few processes that are regulated by miR-21[2,45,46]. For miR-31 a decent number of cancer related studies have been published[47] indicating a more aggressive disease of colorectal cancer[48] if highly expressed. However, a relation to metastastic disease[47,49] and an inverse meaning of increased expression status has been shown in other cancer entities such as breast cancer[48]. The presence of higher expression in different cancer types was reported for miR-135b as well. Nonetheless, it was predominantly analyzed in colorectal cancer and its overexpression by APC loss, PTEN/PI3K pathway deregulation, and SRC overexpression was demonstrated to promote tumor transformation and progression[50]. An oncogenic functionally relevant expression has also been found for miR-223 showing a wide range of different tumor entities[51,52]. In contrast to previous miRNAs, data on the function of miR-492 and its oncogenic relevance are rare. Downregulation of miR-29c - a member of the miR-29 family - is known in several cancer types and its role as a tumor suppressor has been established[53]. Furthermore, its relevance as antifibrotic miRNA is under debate[54]. Initial functional relevance of miR-375 was found as a pancreatic islet-specific miRNA. Recently, miR-375 has been found significantly downregulated in multiple types of cancer, targeting several important oncogenes like AEG-1, YAP1, IGF1R and PDK1[55]. miR-145 is presumed to be a tumor suppressor with apoptosis inhibitor 5, ERK5, K-RAS, and insulin receptor substrate 1 as predicted targets, which are cell cycle and survival regulators[56]. Data on miR-147 is rare; one study postulates that miR-147 is induced upon Toll-like receptor stimulation and regulates murine macrophage inflammatory responses[57]. Taken together, the identified miRNAs from both studies revealed functionally characterized regulators that have, in the vast majority, no organ specificity.

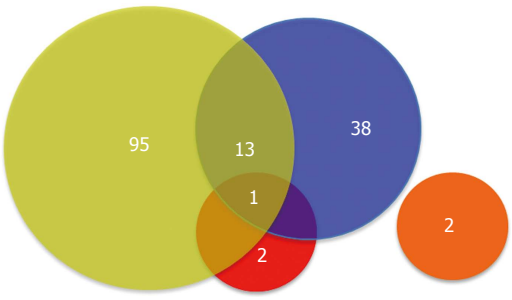
CORRELATION OF MIRNA EXPRESSION TO CLINICAL PARAMETERS

Currently, the most reliable tumor marker to assess clinical outcome is the staging system by TNM classification. As this classification is now more than 100 years old, molecular features for different tumor entities are increasing in number markers for a more precise prognosis are expected. In this respect the aforementioned study of Gaedcke *et al*[38] identified miR-135b. Its expression correlated significantly with disease-free and cancer-specific survival in an independent cohort of 116 patients. miR-135b was also found by other groups to be of importance. Xu *et al*[58] used frozen tissues, performed qPCR analysis, and found miR-135b to have the highest fold-change (17.7-fold) among the upregulated miRNAs in Duke stage Ⅳ cases (that are known to be of poor prognosis). They also identified miR-145 to be highly downregulated with a negative fold change between 18 and 23 in stages Ⅱ, Ⅲ and Ⅳ CRC respectively. Furthermore, they identified significantly decreased expression miR-374a for the identification of patients without metastasis, its effectiveness was confirmed with a sensitivity of 93.33% but a low specificity of only 66.67%. miR-4634 was related to lymph node metastasis in stage Ⅲ with a sensitivity of 75% and specificity of 83.33%. In this analysis, however, the limitation of a mixed study population of colon and rectal cancer must be acknowledged.

Slattery *et al*[59] analyzed data from 1141 CRC cases *via* microarray to identify the impact of 121 miRNAs on disease stage and survival. Five miRNAs were associated with advanced disease stage: hsa-miR-145-5p and hsa-

Li *et al*[37] 2012			
miR-17/18a/18b	Upregulated	miR-1274a	Upregulated
miR-21	Upregulated	miR-10a	Upregulated
miR-31	Upregulated	miR-33a	Upregulated
miR-135b	Upregulated	miR-93	Upregulated
miR-223	Upregulated	miR-98	Upregulated
miR-492	Upregulated	miR-452	Upregulated
miR-19a/20a/a*	Upregulated	miR-886-3p	Upregulated
miR-886-5p	Upregulated	miR-27a	Upregulated
miR-374a	Upregulated	miR-1308	Upregulated
miR-1259	Upregulated	miR-9*	Upregulated
miR-32	Upregulated	miR-106a	Upregulated
miR-196b/196b*	Upregulated	miR-421	Upregulated
miR-96	Upregulated	miR-424	Upregulated
miR-190	Upregulated	miR-1201	Upregulated
miR-182	Upregulated	miR-7	Upregulated
miR-362-3p	Upregulated	miR-183	Upregulated
miR-335	Upregulated	miR-15a	Upregulated
miR-552	Upregulated	miR-106a	Upregulated
Let-7f	Upregulated	miR-200a	Upregulated
miR-301a	Upregulated	miR-339-5p	Upregulated
miR-491-3p	Upregulated	miR-24-2*	Upregulated
miR-140-5p	Upregulated	miR-148a	Upregulated
miR-181d	Upregulated	miR-15b	Upregulated
miR-16	Upregulated	miR-92a	Upregulated
miR-203	Upregulated	miR-25	Upregulated
miR-126	Upregulated	miR-10a	Upregulated
miR-142-3p	Upregulated	miR-30c-1*	Upregulated
miR-374b	Upregulated	miRPlusE1016	Upregulated
miR-29*/a*/b	Upregulated	miRPlusA1087	Upregulated
miR-141*	Upregulated		

Li *et al*[37] 2012	
miR-29c	Downregulated
miR-145	Downregulated
miR-147b	Downregulated
miR-375	Downregulated
miR-1973	Downregulated
miR-378/378c	Downregulated
miR-7-2*	Downregulated
miR-513a-5p	Downregulated
miR-640	Downregulated
miR-381	Downregulated
miR-133b	Downregulated
miR-186*	Downregulated
miR-494	Downregulated
miR-125b	Downregulated
Let-7b/d	Downregulated
miR-139-5p	Downregulated
miR-1184	Downregulated
miR-155	Downregulated
miR-320a-d	Downregulated
miR-150	Downregulated
miR-518e/519a	Downregulated
miR-519b/519c	Downregulated
miR-522/523	Downregulated
miR-125a-5p	Downregulated
miRPlus-E1067	Downregulated
miRPlus-F1027	Downregulated
miRPlus-E1033	Downregulated
miRPlus-E1238	Downregulated
miRPlus-E1173	Downregulated
miRPlus-1285	Downregulated
miRPlus-F1026	Downregulated
miRPlus-E1101	Downregulated
miRPlus-F1155	Downregulated
miRPlus-F1215	Downregulated
miRPlus-E1172	Downregulated
miRPlus-C1110	Downregulated
miRPlus-F1215	Downregulated
miRPlus-E1192	Downregulated



Wang *et al*[39] 2012	
miR-34a	Upregulated
miR-200c	Upregulated

Wang *et al*[40] 2009	
miR-145	Downregulated
miR-143	Downregulated
miR-31	Upregulated

Gaedcke *et al*[38] 2012			
miR-17	Upregulated	miR-29c	Downregulated
miR-18a	Upregulated	miR-143/-145	Downregulated
miR-21	Upregulated	miR-147b	Downregulated
miR-31	Upregulated	miR-375	Downregulated
miR-135b	Upregulated	miR-1	Downregulated
miR-223	Upregulated	miR-30b/c/e	Downregulated
miR-492	Upregulated	miR-26a/b	Downregulated
miR-135b	Upregulated	miR-190	Downregulated
miR-144	Upregulated	miR-192/192*	Downregulated
miR-483-5p	Upregulated	miR-195	Downregulated
miR-542-5p	Upregulated	miR-338-3p	Downregulated
miR-584	Upregulated	miR-10	Downregulated
miR-652	Upregulated	miR-100	Downregulated
miR-675	Upregulated	miR-101	Downregulated
miR-1077	Upregulated	miR-let7a/f	Downregulated
miR-2110	Upregulated	miR-378	Downregulated
miRPlus-E1234	Upregulated	miR-215/194	Downregulated
miRPlus-E1245	Upregulated	miR-429/200b	Downregulated
miRPlus-E1079	Upregulated	miR-148a	Downregulated
miRPlus-A1027	Upregulated	miR-342-3p	Downregulated
		miRPlus-E1141	Downregulated

Figure 1 Differential expression of microRNAs in rectal cancer. The differentially expressed microRNAs (miRNAs) in rectal cancer compared to normal rectal tissue are listed, sorted by studies, respectively. The correlating circles show the number of differentially expressed miRNAs in the mentioned studies and point out the number of miRNAs overlapping between those studies.

miR-31-5p were increased and hsa-miR-200b-3p, hsa-miR-215 and hsa-miR-451a were decreased in advanced stages of CRC. In rectal cancer, 13 miRNAs were significantly associated with mortality after a diagnosis with rectal cancer (Table 1). In addition, they showed that miR-21 expression had an inverse association with mortality in rectal cancer (but not colon cancer patients). However, Nielsen *et al*[60] used *in situ*-hybridization and real-time qPCR on FFPE tissue, and identified miR-21 to predict a short disease-free survival in colon cancer, but not in rectal cancer. Interestingly, the *in-situ*-hybridization showed that the miR-21 expression was detected predominantly in the stromal compartment of the tumors. Yang *et al*[61] showed in an microarray analysis of samples from 40 patients a significant overexpression of miR-21, miR-155, miR-29a and miR-92a in rectal cancer samples and found only miR-155 had the capacity to discriminate nodal positive from negative cases as well as Duke A/B stages from Duke C/D stages.

Stratmann *et al*[62] did not investigate miRNAs directly but the expression level of Dicer - one of the key enzymes in the miRNA generating process - and revealed that the Dicer expression in rectal cancer is higher than in normal mucosa (and higher than in colon cancer), while Dicer expression in liver metastases was decreased in comparison to either the primary tumor or mucosa. Furthermore, patients with a high expression of Dicer mRNA in the normal mucosa had a worse prognosis (poor survival) than those with a lower expression level.

ALTERATION DUE TO THERAPY AND PREDICTING THERAPY RESPONSE

While imaging techniques (computer tomography, magnetic resonance imaging and ultrasound) manage to diagnose tumor stage, nodal stage or distant metastasis initially in an appropriate manner, their ability to identify the response after chemoradiotherapy is poor, particularly the differentiation between vital tumor cells and scar tissue is challenging for imaging techniques. Response to neoadjuvant chemoradiotherapy measured as TRG is therefore usually determined by pathologists after investigating the operative specimen. An adequate

Table 1 Association of microRNA expression and clinical parameters

microRNA	Clinical parameter	Change of expression	Ref.
miR-17-5p miR-20a/20b-5p miR-21-3p/5p miR-25-3p miR-29a/29c-3p	Association with rectal cancer survival	No further explanation	Slattery *et al*[59]
miR-31-5p	Association with advanced tumor stage	Increased expression level in advanced tumor stage	Slattery *et al*[59]
miR-135b	Correlation with disease-free and cancer-specific survival	Patients with a high expression level of miR-135b had a better disease-free and cancer-specific survival	Gaedcke *et al*[38]
miR-135b	Correlation with Duke stage Ⅳ	Upregulated with the highest fold-change (17.7-fold) among 9 upregulated miRNAs	Xu *et al*[58]
miR-141-3p	Association with rectal cancer survival	No further explanation	Slattery *et al*[59]
miR-145	Correlation with Duke stage Ⅱ, Ⅲ, Ⅳ	Downregulated with a -18.15, -18.9, -23.8-fold change in stage Ⅱ, Ⅲ and Ⅳ CRC respectively	Xu *et al*[58]
miR-145-5p	Correlation with advanced tumor stage	Increased expression level in advanced tumor stage	Slattery *et al*[59]
miR-155	Correlation with nodal stage and Duke stage	Discrimination of nodal positive from negative cases as well as Duke A/B stages from Duke C/D stages	Yang *et al*[61]
miR-200b-3p	Association with advanced tumor stage and survival	Decreased expression level in advanced tumor stage	Slattery *et al*[59]
miR-215	Association with advanced tumor stage and survival	Decreased expression level in advanced tumor stage	Slattery *et al*[59]
miR-335-5p	Association between any miRNA expression and survival	The expression of miR-335-5p is associated with a better survival	Slattery *et al*[59]
miR-374a	Correlation with metastasis stage	Decreased expression of miR-374a in tumor of patients without metastasis	Xu *et al*[58]
miR-425-5p	Association with rectal cancer survival	No further explanation	Slattery *et al*[59]
miR-451a	Association with advanced tumor stage	Decreased expression level in advanced tumor stage	Slattery *et al*[59]

CRC: Colorectal carcinoma.

evaluation of response before surgery could spar patients with complete response the surgical resection of the rectum (with all the associated disadvantages), but until today there is no validated biomarker for that. Moreover, since patients respond differently to CRT, a biomarker to predict response to neoadjuvant chemoradiotherapy in rectal cancer patients even before CRT could spar the non-responders the CRT. Understandably, there is a great interest to use miRNAs as possible biomarkers to predict therapy response. Some studies analyzed descriptively the changes in miRNA expression after chemoradiotherapy while others were able to identify miRNAs in tumor tissue, which seem to predict the response to therapy.

Svoboda *et al*[63] performed microarray analysis on tumor biopsies of 31 patients with locally advanced rectal cancer before and 2 wk after chemoradiotherapy with capecitabine (a 5-FU prodrug). They found a significant increase of miR-125b and miR-137 expression levels after 2 wk of chemoradiotherapy. Moreover, they also demonstrated that high levels of miR-125b and miR-137 are associated with a worse response to chemotherapy. However, the sample size is quite short (31 patients), and there is an intertumoral variability described, which should not be neglected. Interestingly, the same group investigated in 2012 in a similar setting 20 patients with locally advanced rectal cancer, whose tumors were classified as most sensible (n = 10) or most resistant (n = 10). They used TaqMan Low Density Arrays analysis to quantify 667 human miRNAs in the tumor tissue samples (preoperative biopsies of untreated primary tumors) and found 8 miRNAs to be significantly differently expressed between the responders and non-responders: miR-215, miR-190b and miR-29b-2 were overexpressed in non-responders while let-7e, miR-196b, miR-450a, miR-450b-5p and miR-99a were down regulated in non-responders[64]; the previously identified miRNAs miR-125b and miR-137 were not mentioned.

Drebber *et al*[65] did real-time-PCR analysis to identify the expression of miR-21, miR-143 and miR-145 in macrodissected FFPE tumor tissue of 40 patients before and after chemoradiotherapy. They described a significant upregulation of miR-143 and miR-145 in post-therapeutic tumor tissue compared to pre-therapeutic tumor tissue. In addition, they showed a significant correlation between a low miR-145 expression in the post-therapeutic tumor tissue and a worse response to CRT. However, this result does not address the problem to predict therapy response in advance: The low expression of miR-145 was measured in the post-therapeutic tumor tissue. To predict tumor response miRNA profiles in the pre-therapeutic tissue are needed.

More adequate to this purpose, Della Vittoria Scarpati *et al*[66] analyzed miRNA expression by microarray and confirmed by qRT-PCR in primary tumor biopsies of patients with locally advanced rectal cancer who underwent neoadjuvant CRT followed by surgery (n = 38). Eleven miRNAs were significantly upregulated in patients with a complete response (miR-1183, miR-483-5p, miR-622, miR-125a-3p, miR-1224-5p, miR-188-5p, miR-1471, miR-671-5p, miR-1909, miR-630, miR-765) and two were downregulated (miR-1274b, miR-720). However, the small cohort of patients' needs additional validation in an independent cohort[66]. Though, none of the mentioned 13 miRNAs was found when Kheirelseid *et al*[67] performed a similar study by using microarray

analysis of 12 FFPE pre-therapeutic tissue samples of rectal cancer to answer to same question by identifying differentially expressed miRNAs. The promising miRNAs in this study were miR-16, miR-590-5p and miR-153 to predict complete *vs* incomplete response and miR-519c-3p and miR-516 to discriminate between good *vs* poor response. Unfortunately, they do not clarify how these miRNAs are altered between the responders and non-responders (downregulated or upregulated).

A possible reason for the different identified miRNAs may be the difference between the tissues used: Della Vittoria Scarpati *et al*[66] used fresh biopsies frozen in liquid nitrogen while Kheirelseid *et al*[67] used FFPE. However, if we act on the assumption that the type of preservation (FFPE, Kryo, *etc.*) differs the miRNA expression, the next question posed would be: What is the preservation effect on miRNA expression and which miRNA expression profile derives from the different tumor characteristics? On the other hand, Hotchi *et al*[68] used also fresh frozen biopsies from 43 rectal cancer patients before starting CRT and did both microarray analysis and RT-PCR of miRNAs concerning response prediction. They found out that miR-223 was higher expressed in tissue from patients with a good response to CRT and declared miR-223 (which is not mentioned by any other study investigating miRNAs in rectal cancer patients for therapy response prediction) as a promising biomarker for the prediction of response to CRT[68]. Other studies found other different miRNAs: Lopes-Ramos found miR-21-5p to be over expressed in tumor biopsies of rectal cancer patients with complete response using fresh biopsies frozen in liquid nitrogen[69], Bhangu *et al*[70] found miR-200c as a possible biomarker to predict CRT response as it shows a significantly reduced expression in non-responders using FFPE material. Figure 2 shows the important miRNAs concerning response to CRT in rectal cancer patients.

In a recent study of our own group, we were able to show with qPCR-analysis a significant decrease of miR-18b and miR-20a during CRT in plasma of patients with a negative nodal stage after CRT (ypN0) compared to those with a positive nodal stage (ypN+). This data presents miR-18b and miR-20a as possible candidates for biomarkers predicting nodal stage after CRT[71]. However, this data requires validation in a lager cohort.

IN VITRO ANALYSES FOR RECTAL CANCER SPECIFIC MIRNAS

Beside the *in vivo* analyses, functional data of specific miRNA that obviously play a role in rectal cancer have been analyzed. One of these is miR-21 that has already been described above. Using tumor biopsies Chang *et al*[72] showed an inverse relationship between miR-21 and programmed cell death protein 4 (PDCD4), a known tumor suppressor[72]. They hypothesized the post-transcriptional modulation of PDCD4 *via* mRNA degradation. These findings were based on data from Asangani *et al*[73], who transfected Colo206f cells with miR-21 and found a significant suppression of PDCD4 proteins *in vitro*.

For miR-182 Amodeo *et al*[74] investigated the effect on thrombospondin-1 (TSP-1), a protein inversely correlated with tumor vascularity and metastasis. In CRC, TSP-1 is shown to be downregulated. After transfection with anti-miR-182, expression level of TSP-1 increased. Hence, the authors concluded that anti-miR-182 could be used to restore TSP-1 expression in CRC to inhibit the angiogenic and invasive events in CRC.

For another rectal cancer associated miRNA, namely miR-455, rapidly accelerated fibrosarcoma (RAF1) seems to be a target gene: In 20 mucosa and 20 CRC biopsies miR-455, miR-484 and miR-101 seem to be down-regulated. An overexpression of miR-455 in SW480 cells showed inhibition of proliferation and invasion. Western Blot analyses showed a downregulation of RAF1 in cells with an overexpression of miR-455, although, on mRNA-level, there was no effect shown[75]. Also the relevance of miRNAs concerning the sensitivity towards CRT could be assessed *in vitro*: Using 12 colorectal cancer cell lines, the miRNA expression profile indicating sensitivity towards an *in vitro* treatment of 5-FU and radiation was established by our own group[76]. These data were validated by the transfection of let7g, miR-132, miR-224 and miR-320a that led to the expected shift of therapy resistance towards sensitivity. For let-7g the higher expression as a good prognostic marker was validated in patient samples.

POLYMORPHISMS IN MIRNAS

Since miRNAs represent one of the important mechanisms of gene expression control, the relevance of polymorphisms concerning miRNAs has been explored in few studies. Naccarati *et al*[77] showed in a case-control study that two single nucleotide polymorphisms within the 3'untranslated regions of target DNA repair genes (nucleotide excision repair genes), hence the miRNA-binding sites, were significantly associated with rectal cancer: rs7356 in RPA2 (predicted binding miRNA: hsa-miR-3149 and hsa-miR-1183) and rs4596 in GTF2H1 (predicted binding miRNA: hsa-miR-518a-5p, hsa-miR-527 and hsa-miR-1205). This study points out that not only the expression levels of miRNAs are relevant, but also their ability to interact with their target gene.

Jang *et al*[78] tried to identify polymorphisms in *miRNA* genes which have a prognostic value in rectal cancer patients and found 196a2C > T (allele of hsa-miR-196a2) polymorphism to be a significant risk factor for the overall survival of rectal cancer patients. The mentioned allele has been reported by other studies to be involved in increased risk of various cancer types[79-81]. Recently, Mao *et al*[82] found miR-146a being decreased in rectal cancer tissue compared to adjacent normal mucosa and they also showed an association between the genetic variant in miR-146a, rs2910164 polymorphism and the risk of CRC.

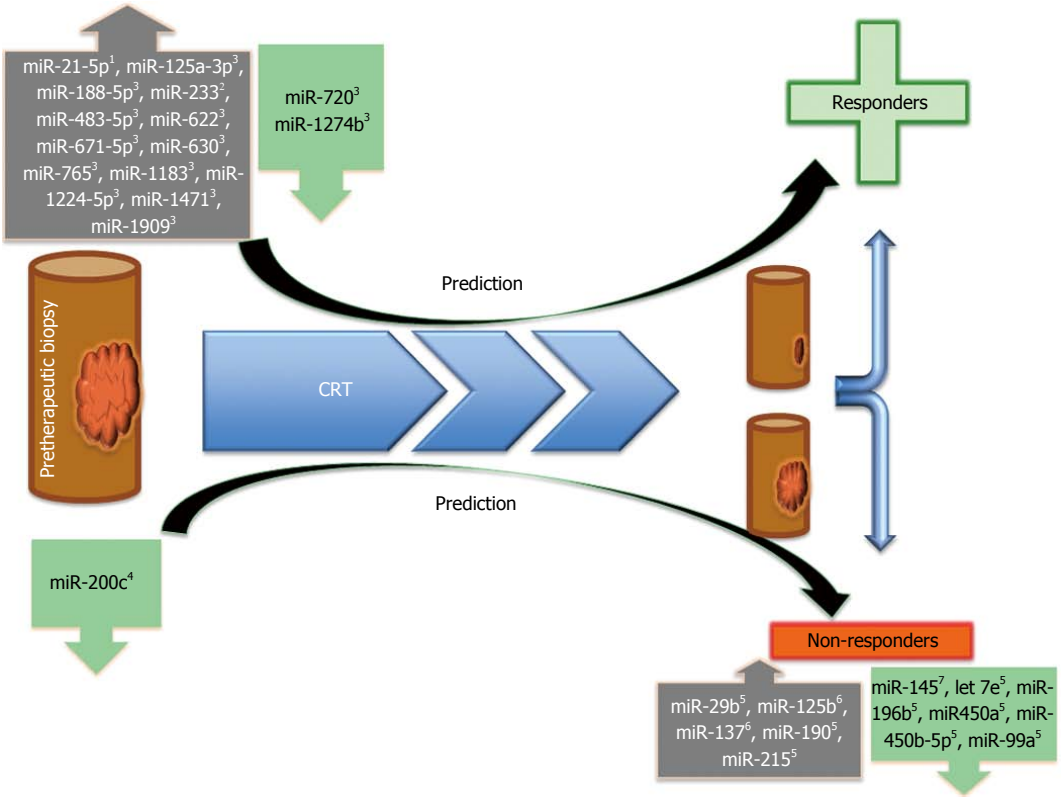


Figure 2 Differential expression of miRNAs dependent on response to preoperative chemoradiotherapy. miRNAs in up arrow callouts are significantly higher expressed; those in down arrow callouts are significantly lower expressed. On the left site there are miRNAs, isolated from pretherapeutic biopsies, which are supposed to predict response or non-response, respectively. The miRNAs in the bottom localized on the right side, are found to be significantly higher or lower expressed in post-therapeutic tumor biopsies of non-responders after chemoradiotherapy compared to pretherapeutic biopsies. [1]Lopes-Ramos *et al*[69], 2014; [2]Hotchi *et al*[68], 2013; [3]Della Vittoria Scarpati *et al*[66], 2012; [4]Bhangu *et al*[70], 2014; [5]Svoboda *et al*[64], 2012; [6]Svoboda *et al*[63], 2008; [7]Drebber *et al*[65], 2011.

CONCLUSION

miRNAs are widely accepted to play a crucial role in physiological and pathological processes. Interestingly, in contrast to the relevance of rectal cancer and its frequency, especially compared to colon cancer, the number of available studies is rather small. The amount of studies as well as the small number of patients per study may be one of the reasons why only few overlapping miRNAs have been identified. Importantly, a small number of miRNAs were identified with relevance in rectal cancer. Many of these are rather known from cancer specific mechanisms than display rectal cancer specificity. Accordingly, the relevance of miRNAs as a predictive or prognostic biomarker in rectal cancer is questionable. Furthermore, the relevance of functional miRNAs does not appear to be as obvious as in previous studies that are typically cell-line based. However, before ignoring the relevance of miRNAs it should be taken into account that human cancer tissue is functionally a rather complex cell system. The analyses are impeded by the heterogeneity of tumor biopsies that in general include different amounts of non-tumor cells such as stroma or the surrounding tissue. Different analyzing techniques applied to identify miRNA (PCR, microarray, *etc*.) or the varying fixation media (FFPE, fresh frozen biopsies, *etc*.) further complicate the comparability of the data. Furthermore, subtle expression differences of a given miRNA that potentially change complex regulatory mechanism simply may not be identified. This may due to the techniques applied or has simply not been part of the analyses that, in general, focus on expression fold changes.

Specifically for miRNAs, there may be alternative reasons for varying results, such as the highly variability of miRNA expression due to external influences such as nutrition. Humphreys for example, showed that the expression of oncogenic miRNAs can be altered by dietary manipulation: A high red meat intake leads to elevated miR-17-92 (cluster) and miR-21 in rectal mucosa tissue of healthy volunteers. While organ specificity is well known for miRNA, Li *et al*[37] identified miRNA expression differences (*e.g*., miR-182) in CRC between African and Caucasian Americans. Possibly, there are further influences like medications used by the patients, gender differences, or age associated variations that are much higher than currently expected.

Overall, there is a large number of possible reasons as to why a clear identification of miRNAs still failed. However, compared to alternative molecular markers in rectal cancer such as proteins, mRNA or DNA, miRNA are not inferior as there are currently no well established markers. Acknowledging some of the previously listed points, miRNA analyses in rectal cancer aiming to identify regulatory mechanisms or to establish marker for

prediction or prognosis should be endorsed. Furthermore, these efforts should be expanded to blood samples as it has been done in many other cancer types.

FUTURE PERSPECTIVES

Validity of cell free and cellular miRNAs as a prognostic or diagnostic tool remains, at least in parts, elusive. The incomplete understanding of biological processes yielding circulating RNAs and their physiological relevance needs to be addressed in more detail, *e.g.*, by application of less bias-sensitive technologies and combinations of, *e.g.*, high-throughput sequencing, qPCR and microarray techniques[83]. Functional characterization of altered miRNAs in CRC and surrounding healthy tissue with respect to more recent findings of modifications that impact miRNA processing and target-gene regulation will improve quality and interpretability of the datasets originating from quantitative analysis[84]. Investigations on differential or coherent expression of miRNAs in affected tissues, changes of strand-selection during tumor progression, and treatment as well as in-deep analyses of the physiological relevance of secreted miRNAs and other non-protein coding RNAs can clarify roles of these and feasibilities to choose particular candidates as markers for prognosis and diagnostics or candidates for therapies[23,36].

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REVIEW

Role of Raman spectroscopy and surface enhanced Raman spectroscopy in colorectal cancer

Cerys A Jenkins, Paul D Lewis, Peter R Dunstan, Dean A Harris

Cerys A Jenkins, Paul D Lewis, Dean A Harris, Swansea University Medical School, Swansea University, Swansea SA2 8PP, United Kingdom

Peter R Dunstan, Department of Physics, College of Science, Center for Nanohealth, Swansea University, Swansea SA2 8PP, United Kingdom

Dean A Harris, Department of Colorectal Surgery, Singleton Hospital, Swansea SA2 8QA, United Kingdom

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Correspondence to: Dean A Harris, MD, FRCS, MB, ChB, Department of Colorectal Surgery, Singleton Hospital, Sketty Lane, Swansea SA2 8QA, United Kingdom. dean.a.harris@wales.nhs.uk
Telephone: +44-1792-285459

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Abstract

Colorectal cancer (CRC) is the fourth most common cancer in the United Kingdom and is the second largest cause of cancer related death in the United Kingdom after lung cancer. Currently in the United Kingdom there is not a diagnostic test that has sufficient differentiation between patients with cancer and those without cancer so the current referral system relies on symptomatic presentation in a primary care setting. Raman spectroscopy and surface enhanced Raman spectroscopy (SERS) are forms of vibrational spectroscopy that offer a non-destructive method to gain molecular information about biological samples. The techniques offer a wide range of applications from *in vivo* or *in vitro* diagnostics using endoscopic probes, to the use of micro-spectrometers for analysis of biofluids. The techniques have the potential to detect molecular changes prior to any morphological changes occurring in the tissue and therefore could offer many possibilities to aid the detection of CRC. The purpose of this review is to look at the current state of diagnostic technology in the United Kingdom. The development of Raman spectroscopy and SERS in clinical applications relation for CRC will then be discussed. Finally, future areas of research of Raman/SERS as a clinical tool for the diagnosis of CRC are also discussed.

Key words: Detection; Colorectal cancer; Spectroscopy; Raman; Surface enhanced Raman spectroscopy

Core tip: This review focuses of the current role of Raman spectroscopy and surface enhanced Raman spectroscopy (SERS) in clinical applications of colorectal cancer. This includes a review of the current research into *in vivo* endoscopic Raman probes, non-destructive analysis of biofluids and the use of SERS in order to detect low concentration analytes that previously could not be detected with Raman spectroscopy. Both the advantages and disadvantages of the technology are discussed along with possible avenues of future research.

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INTRODUCTION

Colorectal cancer (CRC) is the fourth most common cancer in the United Kingdom and is the second largest cause of cancer related death in the United Kingdom after lung cancer[1]. Currently in the United Kingdom there is not a diagnostic test that has sufficient differentiation between patients with cancer and those without cancer so the current referral system relies on symptomatic presentation in a primary care setting[2,3]. CRC results from the progressive accumulation of genetic and epigenetic alterations that disrupt normal cellular mechanisms[4]. The 5-year survival rate for CRC detected in early stages are > 90%, however the 5-year survival rate for later-stage cancers is < 10%[1,5]. This highlights the need for a simple, reliable diagnostic test that can detect early signs of the disease. The majority of CRCs present symptomatically in a primary care setting so it is important that general practitioners can identify patients who are at highest risk[2]. The risk associated to patients is based on the referral guideline, in the United Kingdom is this based on a combination of symptoms and age of the patient[6]. The relationship between initial symptoms and mortality as a diagnostic indicator have previously been discussed in depth[2,3,7,8]. Unfortunately initial symptoms associated with CRC can also be symptoms of benign diseases such as irritable bowel syndrome[6], and there is currently no diagnostic test available in primary care that has sufficient differentiation to base referral on[3]. Furthermore the detection of CRC using symptoms has been shown to be ineffective for decreasing mortality rates in comparison to the European average[9].

Raman spectroscopy and surface enhanced Raman spectroscopy (SERS) could hold many advantages for use as a diagnostic tool for CRC. These techniques have previously been used to discriminate between cancerous and non-cancerous tissue, biofluid samples, as well as in the development of *in vivo* Raman systems for use in endoscopy. The techniques are non-destructive to samples so can provide molecular information about a sample without the need for staining or in some cases without the need for resection. Raman and SERS have the potential to detect molecular changes in cancerous cells/tissues and biofluids that precede morphological changes such as the development of precursor lesions. The techniques could offer the potential for an early detection tool that detects molecular changes before the stage at which a traditional histopathology would be able to detect. This review paper will discuss the previous applications of Raman spectroscopy and SERS to detect CRC.

CURRENT DIAGNOSTIC PATHWAYS

In United Kingdom, CRC survival outcomes are lower than the European average, this is be attributed to a higher proportion of cancer being diagnosed at late-stage[9]. In order to combat the NHS rolled out a national screening program in 2006. There are currently two types of screening method available to screen for CRC and late-stage adenoma namely flexible sigmoidoscopy (FS) and measurement of markers in faecal samples[10]. FS is a procedure involving the insertion of an endoscope into the rectum of a patient in order to examine the distal colon and rectum; the procedure must be carried out by a trained doctor. Atkin *et al*[11] (2010) conducted a United Kingdom study into the effectiveness of a single FS procedure as a screening tool. A procedure for patients aged 55-64 years the study saw a 33% reduction in CRC cases and a 43% mortality reduction, however this is only applicable with respect to the distal colon[11]. However, the cost of a FS is high in comparison to tests that look for markers in faecal samples because it involves trained staff[12]. Moreover, it can be argued that the results of tests reliant on the opinion of a practitioner can be subjective depending on the experience of the practitioner.

In England and Wales the guaiac faecal occult blood test (gFOBT) is commonly used as a screening tool. The test is simple and cheap compared to other methods[12]. Problems that occur with gFOBT regarding dietary requirements of patients before taking the test are solved using immunochemical feacal occult blood testing (iFOBT)[13]. There are different types of iFOBT, all of which detect human specific haemoglobin in faecal samples. Investigations and meta-analysis studies have shown iFOBT to have improved sensitivity in the detection of CRCs and late-stage adenomas in both high-risk, average-risk and populations with no overt rectal bleeding compared to gFOBT without compromising on specificity[14-17]. However, despite the improved sensitivity of iFOBT tests patient uptake of the screening programme in general is poor. In October 2008 of the 2.1 million people that had been invited to partake in the screening programs in England uptake was just 55%-60%[18]. Other diagnostic methods available to clinicians in the United Kingdom include double barium enema, computed tomography colonoscopy, MRI imaging and colonoscopy. Outside of the United Kingdom there are other diagnostic tests are also in use such as the tumour M2-pyruvate kinase (tumour M2-PK) test. This is a faecal test that investigations have shown this to also be more sensitive than FOBT[19]. However, the cost effectiveness and the sensitivity and specificity of the M2-PK especially compared to the iFOBT still needs to be established in the United Kingdom as published results are in disagreement as to whether it is more effective than iFOBT[20]. There is also a real time polymerase chain reaction based blood test that is available outside of the United Kingdom that detects methylated Septin 9 (mSept9). The blood test

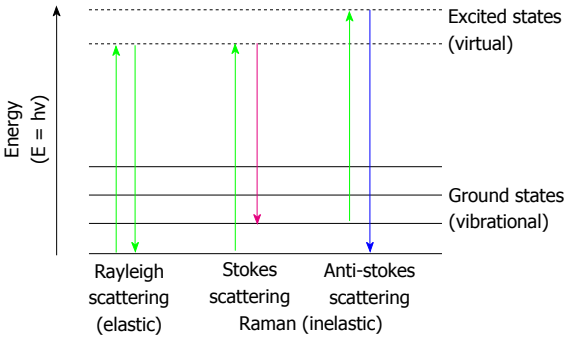

Figure 1 Energy shifts involved in light scattering interactions. Adapted from Lin *et al*[23].

has been shown to have sensitivity and specificity ranging from 50%-90% and 88%-91% respectively[21]. A blood test is a potentially more attractive option for patients compared to faecal and colonoscopy tests so studies are underway to determine if the higher cost of mSept9 would be recovered by higher screening uptake[22].

Raman spectroscopy could offer a highly sensitive and less invasive alternative/complimentary technique to aid CRC detection. However, Raman spectroscopy has not yet found its way into a routine clinical setting. This review will examine the current research status of the application of Raman spectroscopy for detecting CRC to critique the prospective translation of the technique to a clinical setting.

Methods for review

A systematic literature search (PubMed, MEDLINE, Web of Science) was conducted using the following terms: "Diagnosis", "Raman spectroscopy", "Surface enhanced Raman spectroscopy", "SERS", "CRC", "Tissue", "Biofluids", "Immunoassay", "CEA". Following these searches 30 studies were selected for inclusion based on the criteria: (1) That they are original studies based on the clinical applications of Raman spectroscopy, SERS or both for CRC detection in human tissue, biofluids or cell lines; and (2) in the case where there are large amounts of similar studies that they were the first to report such data. Only studies published up to January 2015 were included in this review.

RAMAN SPECTROSCOPY AND SERS FOR THE DETECTION OF CRC

Introduction

Raman spectroscopy is a type of vibrational spectroscopy that allows the user to gain molecular information about a sample through the scattering of incident light. In general, when light is passed through or onto a sample a small proportion of the photons are scattered (approximately 10^{-5}). The majority of this is Rayleigh or elastic scattering; where the energy of the incoming photon is equal to the energy of the scattered photon (Figure 1)[23]. Around 1 in 10^7 of the incident photons are in-elastically scattered resulting in the incident photon and the scattered photon having a difference in energy.

The inelastic scattering is a relatively weak effect which was first observed in 1928 by Sir CV Raman and is known as Raman scattering[24]. When scattered light is measured with a spectrometer a series of lines are observed, the shift in the energy [measured by wave-number (cm^{-1})] from the Rayleigh line (equal to incident energy) is known as the Raman shift. The shift recorded corresponds to specific vibrational or rotational modes of the sample molecule. The intensity of the "Raman shift" for a particular molecule is directly proportional to the concentration of that molecule within a sample so the resulting spectrum of a sample will be a superposition of Raman response of all the Raman active molecules from within a sample, *e.g.*, proteins, nucleic acids, *etc*. It is interpreted as the molecular "fingerprint" of the sample, an example of a spectrum can be seen in Figure 2. It is due to the "fingerprint" that Raman is seen to be desirable for application to cancer detection because it offers the possibility of detecting minute differences in analyte concentrations and is a non-destructive technique.

Vibrational techniques

It should be noted that Raman is not the only type of vibrational spectroscopy that has been reviewed for clinical applications[25,26]. One of the other main areas of vibrational spectroscopy being applied to clinical applications is fourier transform infrared spectroscopy (FTIR) and a type of enhancement infrared attenuated total reflection. FTIR relies on either the absorbance or the transmission of light through a sample, then similar to Raman the difference in the emitted and absorbed or transmitted light is measured to gain molecular information about a sample. This technique has also been used for diagnosis of CRC independently and also coupled with immunohistochemical staining[27,28]. Compared with other vibrational spectroscopy techniques Raman holds many desirable properties for the application to a screening method. One of the biggest advantages of using Raman spectroscopy is that samples can be in aqueous solutions due to water having a small Raman cross-section at near-infrared wavelengths, water has a high absorbance in FTIR and therefore can interfere with a spectrum. Table 1 shows a comparison table constructed from literature to give an overview of the strengths and weaknesses of FTIR, Raman and traditional hematoxylin and eosin staining (H and E) when used for clinical applications[25,29,30]. It suggests that for the application to biological samples such as tissues and biofluids Raman could be the most favorable option. Like FTIR it is non destructive and can be performed in real time compared to H and E staining. However, Raman spectroscopy has the advantage over FTIR of being able to scan over a larger wavenumber range than FTIR and with better spatial resolution than both FTIR and H and E. Furthermore, Raman is a technique that relies on scattering so measurements can be taken with single ended endoscopic probes. This is advantageous for *in vivo* applications because it makes it possible to study

Table 1 A comparison of Raman spectroscopy, fourier transform infrared spectroscopy and hematoxylin and eosin staining strengths and weaknesses

	Raman spectroscopy	FTIR	Hematoxylin and eosin staining
Method of detection	Inelastic scattering of monochromatic (laser) light	Absorbance (polychromatic light source)	Combination of basic and acidic dyes
Real time	Yes	Yes	No
Wavenumber range (cm^{-1})	50-4000	400-4000	N/A
Spatial resolution	1 μm	5 μm	Cellular
Enhancement techniques	SERS, TERS, CARS, SORS, SRS	ATR	"Special" staining
Effect of water	Minimal	Large absorbance in NIR region	No
Destructive to sample	No	No	Yes

SERS: Surface enhanced Raman spectroscopy; TERS: Tip enhanced Raman spectroscopy; CARS: Coherent anti-Stokes Raman spectroscopy; SORS: Spatially offset Raman spectroscopy; SRS: Stimulated Raman spectroscopy; ATR: Attenuated total reflection; FTIR: Fourier transform infrared spectroscopy; NIR: Near-infrared; N/A: Not available.

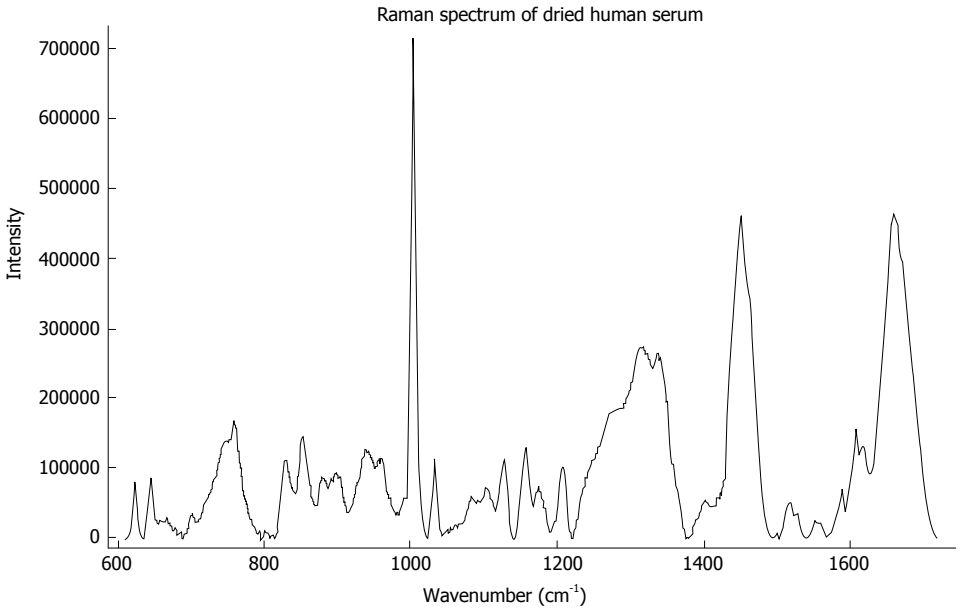

Figure 2 Example Raman spectrum of dried human serum. Spectrum of dried droplet of human serum taken with 785 nm laser excitation, 10 s acquisition time with an InVia Raman spectrometer (Renishaw, United Kingdom).

samples that are optically too thick for transmission techniques[31]. This, along with the need for a less invasive diagnostic tool that can analyse liquid biofluids leads to the remainder of this review focusing on the application of Raman and SERS technology.

Data manipulation

Many clinical applications of Raman and SERS use chemometic data analysis techniques in order to aid data manipulation and to pick out the small differences that could indicate disease. Details of principle component regression (PCR) and partial least squares can be found in Kramer (1998)[32]. Principle component analysis (PCA) applications can be found in Shinzawa *et al*[33] (2009). This review will only state the technique that has been used in each study.

CLINICAL APPLICATIONS OF RAMAN SPECTROSCOPY IN CRC

Histopathological analysis of tissue biopsies is still considered the gold standard for the diagnosis of malignant tissues that have been surgically resected. Typically, thin sections of tissue are "fixed" usually using formalin and then mounted onto glass slides and stained using various methods in order to determine TNM stage, tumour type, histologic grade and the level of vascular invasion. However, histopathology is a slow process that requires a trained pathologist, it is also inherently subjective[34]. Raman spectroscopy offers the possibility of determining the presence of malignancy by detecting differences in Raman spectral features between normal and malignant tissue. Previously, Raman spectroscopy has been applied to *in vivo* probes that have the ability to discriminate multiple tissue types[35,36], biofluid analysis[37] and also analysis of cancerous cell lines for both discrimination and characterization[38,39]. The motivation behind using Raman used *in vivo* is to aid rapid diagnosis and help to identify possible areas of tissue for biopsy that might otherwise be missed. A summary of the literature and different applications of Raman towards clinical applications for CRC can be found in Table 2.

Table 2 A summary of the different clinical applications of Raman spectroscopy to colorectal cancer

Method	Sampling type	Sample number	Ref.	Year	Spectral region (cm^{-1})	Laser excitation (nm)	Data analysis
Probe	*In vivo* (tissue)	20	Shim *et al*[42]	2000	450-1800	785	PCA, PLS, ANN
Probe	*In vivo* and *ex vivo* (tissue)	9	Molckovsky *et al*[44]	2003	900-1800	785	PCA, LDA, LOOCV
Micro-spectrometer	*In vitro* (primary tissue)	10	Chen *et al*[38]	2006	500-1900	782.5	PCA
Probe	*Ex vivo* (tissue)	59	Widjaja *et al*[45]	2008	800-1800	785	PCA, SVM, LOOCV
Micro-spectrometer	*Ex vivo* (tissue)	54	Beljebbar *et al*[47]	2009	600-1800	785	SVM, PCA
Micro-spectrometer	*In vitro* (serum)	120	Li *et al*[37]	2012	800-1800	785	PCR, PLSR, LDA
Micro-spectrometer	*In vitro* (cell lines)	N/A	Ranc *et al*[39]	2013	400-1800	532	PCA
Probe	*In vitro* (tissue)	177	Wood *et al*[43]	2014	800-1800	830	PCA, LDA, LOOCV
Micro-spectrometer	*In vivo* (tissue)	50	Bergholt *et al*[78]	2014	800-1800 and 2900-3600	785	PLS, LDA

PCA: Principle component analysis; LDA: Linear differential analysis; PLS/PLSR: Principle least squares regression analysis; LOOCV: Leave one out cross validation; ANN: Artificial neural network; SVM: Support vector machine; PCR: Principle component regression.

Table 2 shows that most clinical applications of Raman have been in the near-infrared (NIR) region using 785 nm laser excitation analysing tissue samples. This is likely due to reduced fluorescence of biological; samples in the NIR region, furthermore 785 nm laser is less powerful than visible region lasers so is less likely to cause damage to biological samples. The work is generally done in the "fingerprint region" of the Raman spectrum, *i.e.*, 400-1800 cm^{-1} due to molecular bonds present in biological samples being Raman active in this region. All of the studies use chemometric data analysis so it seems for NR this is essential to differentiate the small differences in the Raman spectra when using biological samples. It is also clear that work in the field has previously been dominated work towards *in vivo* Raman probes for use during endoscopy but more recent work has used both Raman probes and microspectrometers.

Tissue analysis

Reviews dedicated to Raman spectroscopy for the use of *in vivo* probes for clinical use for many types of tissue including tissues of the gastrointestinal (GI) tract are already available[25,40,41]. Briefly, the *in vivo* probes for use during endoscopy for use with GI tissue was first introduced by Shim *et al*[42] (2000). In general, the *in vivo* probes use a laser excitation source in the NIR wavelength range (light is non-mutagenic in this region) coupled to an optical fibre probe. The probe can then act as both a source of light and a detector relaying a signal back to a charged coupled device detector and computer analysis. The development of the probes is not a simple process, some materials that the probes are manufactured from have a large Raman cross sections leading to design challenges regarding signal to noise ratio (gaining unwanted noise from the material). Other issues can be caused by tissue fluorescence signal being larger than the Raman signal making data acquisition difficult and spectral acquisition times to be impractical for clinical applications (more than 10 min). Nevertheless, some research groups have been successful in designing probes for specific use with gastrointestinal tissue for use in routine endoscopy that have short acquisition times[35,43].

Shim *et al*[42] (2000) successfully applied an *in vivo* probe to gain Raman spectra of colonic and oesophageal tissues from 20 patients. The pressure spectral acquisitions were made with 5 s exposure time and repeated at normal and malignant sites within both areas of interest. In the colonic tissue subtle differences between spectral area 1100-1800 cm^{-1} were identified however no significant prominent changes were evident. PCA and LDA analysis was then introduced in order to try and distinguish accuracy for application to GI diagnostics however no specific results relating to diagnostics were published.

Molckovsky *et al*[44] (2003) were the first to assess the diagnostic potential of near infrared Raman spectroscopy on colonic tissue by using adenomatous polyps as a model for dysplasia. The group used a custom-made fiber-optic Raman probe and used PCA-LDA analysis and LOOCV to analyse a total of 33 polyps from 8 patients. After an initial *ex vivo* study of polypectomy specimens that involved a total of 54 spectra the analysis algorithm identified a sensitivity of 91% and a specificity of 95%. An *in vivo* study was then conducted with a total of 19 spectra form 9 polyps, after spectral analysis the algorithm identified adenomas with a sensitivity of 100% and specificity of 89%. A similar study involving a higher number of specimens for a similar use on *ex vivo* tissues was conducted by Widjaja *et al*[45] (2008), the group were able to differentiate cancerous tissue with 100% sensitivity and 98.1%-99.7% specificity using a diagnostic algorithm using PCA and LDA.

Raman spectroscopy has also been investigated as a complimentary technique to histopathology. The first application of Raman spectroscopy for characterisation of colonic tissue (among others) to discriminate between cancerous *vs* normal was by Feld *et al*[46] (1995). The group looked at the difference spectra between normal and cancerous tissue, this results showed potential that spectral differences in the tissue could be due to higher nucleic acid levels in the cancerous samples[46].

Beljebbar *et al*[47] (2009) used a Raman micro

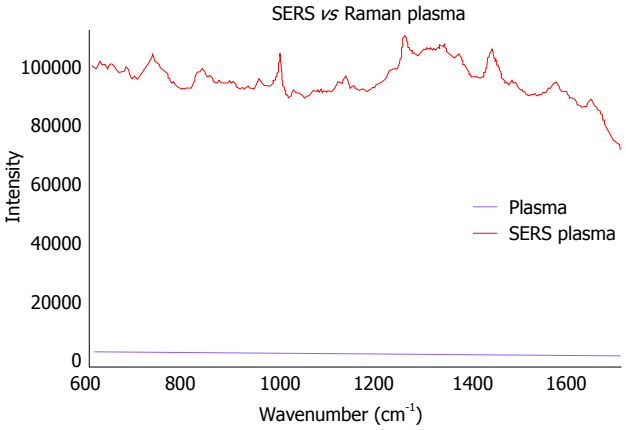

Figure 3 An example spectrum of a surface enhanced Raman spectroscopy response *vs* Raman response of human blood plasma. Raman spectrum and SERS spectrum of dried human plasma droplet at 10% laser power with 785 nm laser excitation, 10 s acquisition time with an InVia Raman spectrometer (Renishaw, United Kingdom). SERS response gained by 1:1 mixing with 40 nm raw gold nanoparticles (Nanocs, United States). Purple: SERS response of plasma; Blue: Raman response of plasma. SERS: Surface enhanced Raman spectroscopy.

spectrometer on 27 normal and 27 cancerous *ex vivo* frozen tissue samples. Unsupervised hierarchal cluster analysis to differentiate between normal and adenocarcinomatous human colonic tissues was discussed. The technique was based on the spatial distribution of molecular changes in colon constituents such as proteins, lipids and nucleic acids. The spectroscopic data was then used to create pseudo-colour Raman images of tissues for comparison with histopathological slides. The data was then used to create databases for the purpose of comparing unknown specimens. Six extra frozen unknown samples specimens were fed into the database and were correctly identified as either cancerous or normal[47]. This study showed the potential for Raman spectroscopy to aid histopathology by adding structural molecular information to the visible information gained from H and E staining. This study used frozen tissue samples but there are different fixation methods available. There have been studies on the effect that different fixation methods of both cell lines and tissue samples taken from resection has on the Raman spectral signature but these will not be discussed further in this review[48-50].

It should be noted that Raman spectroscopy used as an adjunct to histopathology has not just been applied to CRC. Some groups have investigated the use of stimulated Raman spectroscopy (SRS) to create spectral histopathological images for breast, brain and skin tissue among others[51-54]. For example, Satoh *et al*[54] (2014) used SRS and PCA multivariate analysis to produce Raman map images of damaged liver tissue in mice. The images could then be compared to different staining methods used in histopathology.

DETECTION OF CRC IN BLOOD SAMPLES

The advantage of Raman spectroscopy over other vibrational techniques is its ability to analyse samples in aqueous solution with minimal background. This is advantageous for the study of samples such as urine and blood as they can be analysed without changing their composition by drying.

Berger *et al*[55] (1999) introduced the idea that Raman had potential for the analysis of biofluids, in particular human blood. Premasiri *et al*[56] (2012) found that the NR spectra of whole human blood are dominated by the normal modes of either oxygenated or deoxygenated haemoglobin porphyrin macrocycle and haemoglobin. This leads to the assumption that if NR is to be used for the analysis of blood components such as malignancy specific proteins for CRC other than those associated with red blood cells (RBC) an enhancement mechanism has to be used or blood samples must have RBC removed in order to overcome this issue (Figure 3).

For example, Harris *et al*[57] (2009) discussed the potential of peripheral blood samples analysed by NR to provide cancer screening in head and neck cancer. Using Raman spectroscopy and LDA analysis alone the study found this technique to have approximately 65% specificity and sensitivity for the discrimination of cancer in peripheral blood samples. The use of non-enhanced Raman spectroscopy for the discrimination of blood serum between normal and CRC was first reported by Li *et al*[37] (2012). Li *et al*[37] presents a study using clinical samples from 44 colon, 46 rectum and 30 healthy controls. Raman peak parameters and fluorescence background were used along with multivariate analysis techniques such as PCR and PLSR for the dimension deduction of spectral data. Then, LDA on PC's is used to see diagnostic performance. Three distinct, Raman peaks were found to have significance at 1029 cm^{-1}, 1538 cm^{-1} and 1170 cm^{-1}. The 1538 cm^{-1} peak was assigned to beta-carotene and 1170 cm^{-1} to tryptophan and phenylalanine. The average spectra from the three sample groups were then compared and the latter two peaks were shown to decrease compared to the control group. This is explained as a decrease in anti-cancer related molecules.

This study showed that it is possible to discriminate between serum samples of patients with and without CRC. The result of the PCR-LDA analysis was promising as it identified normal samples with 87.5% accuracy and 96.7% specificity and colon cancer samples with a sensitivity of 84.8%.

SERS detection methods

Raman spectra are subject to interference from samples that exhibit fluorescence; a fluorescence signal is far greater than the Raman response so it can "hide" any Raman signal. Fluorescence is fairly common when using wavelengths less than 785 nm that are commonly used in biological studies. Also, when Raman is used on complex biological samples Raman peaks can be additive, making differentiation between biological markers difficult to resolve, this results in a reliance on data manipulation in order to detect small spectral differences. SERS offers a resolution to some of these

issues as it reduces the effects of inherent fluorescence while increasing the intensity of the Raman response of the sample. Premasiri *et al*[58] (2001) demonstrated the need for an enhancement mechanism in order to detect some low concentration analytes in urine analysis by Raman spectroscopy. It was found that urea had a sufficiently high concentration to be analysed by NR in liquid form however, lower-level nitrogen compounds needed enhanced Raman spectroscopy in order to detect these compounds[58]. Therefore SERS was used as an enhancement mechanism in order to be able to detect the compounds that were in concentrations too small to detect with Raman.

The basic principle of SERS is to amplify the Raman response of a given analyte. The SERS effect was first discovered in 1974, and understood to be an enhancement of Raman scattering in 1977[59,60]. This is generally achieved by having an analyte attached or close to the surface of a nanoscale metal substrate causing an enhancement factor of up to 10[14,15,61,62]. The exact mechanism of SERS enhancement is still an area of active research, however it is generally accepted that two mechanisms contribute to the enhancement[63]. One is based on electromagnetic field enhancement due to excitation of electromagnetic resonances in the SERS active nanoscale metal substrate. The other is known as "chemical enhancement" which is a result of the metal electrons causing a charge transfer between the metal substrates and the adsorbates. The result of the combined enhancement is an extremely powerful technique that combines ultra sensitive detection limits with the molecular structure information from Raman spectroscopy giving the possibility of single molecule detection[64].

Clinical applications of SERS for CRC

The main use of SERS in clinical applications for CRC has been as a detection method. Lin *et al*[65] (2011) were the first to report SERS serum analysis for the detection of CRC. In their study colloidal gold nanoparticles were simply mixed with serum samples from 38 patients and 45 control samples and dropped onto an aluminium substrate. This technique is known as label-free SERS. It generally relies on blood constituents being adsorbed onto the surface of metallic nanoparticles causing an enhanced Raman response. In the Lin *et al*[65] study a Raman micro-spectrometer (Renishaw, Great Britain) fitted with a 785 nm diode laser was used to gain spectra in the 300-1800 cm^{-1} range. Spectra from the two groups were normalised to the integrated area under the curve in the 350-1750 cm^{-1} wavenumber range. The mean spectrum for the normal serum and the cancer serum were then compared to isolate wavenumbers that showed the most variation between the two groups. Then an empirical diagnostic algorithm based on peak intensities at 725 cm^{-1} and 638 cm^{-1} was used to classify the normal and the cancer samples. These were chosen based on previous studies by Han *et al*[66] (2008) that showed the ratio to be important disease marker. This technique was compared PCA-LDA multivariate approach. The PCA analysis then used whole spectra to discern the spectral components that had the largest variation. After comparison the group found PCA-LDA to be more effective at detecting CRC. With specificities for detecting cancer to be 68.4% and 97.4% for the empirical approach and the multivariate approach respectively.

This study showed that through simply mixing gold nanoparticles with serum that it is possible to discriminate between normal and cancer samples using SERS along with both empirical and multivariate analysis techniques. The potential for using whole spectra coupled with PCA-LDA to be used as a screening technique for CRC was then discussed using the PCA-LDA methods in a second publication from the same group[65]. The two publications also included tentative peak assignments and major vibrational bands that have been previously observed in serum samples. The peak assignments are vital to being able to accurately describe what is happening at the molecular level when a patient has a disease. However, due to the additive nature and complex compounds found in serum samples it can sometimes be difficult to be sure of peak assignments. Furthermore, the methods described above such as looking at the 725 cm^{-1} band can be a marker for disease but it is not specific to CRC. In order for the technology to become useful as a diagnostic there is a need to have a Raman/SERS marker that is specific to CRC.

The need for specific detection has motivated the development of "labelled" SERS probes. These probes have previously been used for detecting disease specific proteins in both tissue and serum samples[67-71]. They have also been used for the detection of circulating tumour cells[72]. However there is little reported on the specific application of targeted SERS probes for use in detecting CRC[73]. In general targeted SERS techniques rely on either aggregation of antibody-functionalised nanoparticles after exposure to a protein or they are used to form of sandwich immunoassay similar to that of an ELISA setup but using SERS active probes rather than fluorescent-tagged antibodies (Figure 4).

Both techniques offer advantages and disadvantages; techniques relying on the aggregation of nanoparticles use fewer antibodies and in general have very simple protocols that can be done on cheap substrates. However, when dealing with the aggregation of nanoparticles it can be difficult to "find" the correct spot on a sample where the SERS intensity is greatest. Furthermore, studies relying on aggregation can be susceptible to large variation as controlling the aggregation can be difficult. SERS based immunoassay holds the advantage over aggregation because if the disease specific protein is in a sandwich style assay then the area to probe is easier to locate and the Raman signal is less likely to be variable, as one would expect more even coverage of the protein over the assay area. One advantage of both of these techniques over current fluorescence methods is that Raman bands are much

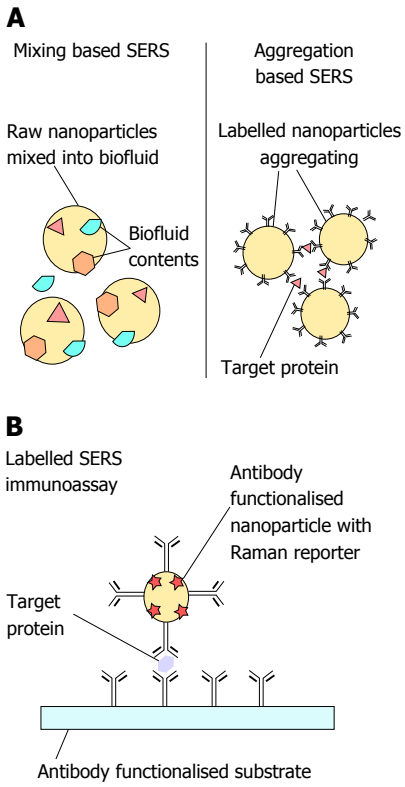

Figure 4 Different methods of producing a surface enhanced Raman spectroscopy response with biological samples. A: Mixing based methods with both non-labeled mixing and labeled or "aggregation" based mixing; B: Labeled surface enhanced Raman spectroscopy based immunoassay. SERS: Surface enhanced Raman spectroscopy.

narrower than those in fluorescence which means there is the potential to use more probes in a multiplex assay than is currently possible with fluorescent probes. Chen *et al*[74] (2013) developed a SERS based immunoassay for the detection of carcinoembryonic antigen (CEA) in serum of patients with CRCs. CEA antibody functionalised glass slides were used in conjunction with SERS active probes that were also functionalised with CEA[74]. A range of concentrations from 5×10^{-3}-5×10^{5} ng/mL CEA were prepared and the SERS response monitored. The SERS intensity was linearly correlated with the concentration of CEA in the characteristic peak or the Raman reporter molecule at 1077 cm^{-1} and hence a calibration curve was established. CEA concentrations in serum samples form 26 patients with CRC were then analysed with both SERS immunoassay and electrochemical luminescence. The results were then compared and it was found that the two techniques had similar agreement. Using the calibration curve for the patient samples a detection limit of 5 pg/mL was achieved. This is the only study specifically using CEA for the detection of CRC, however there is other work in the literature using CEA conjugated antibodies but towards use for other diseases[69,70].

Ito *et al*[75] (2014) developed a SERS based assay that used silver nanoscale hexagonal columns (NHC) on phosphor bronze chips. The chips were negatively ionized and 36 clinical serum samples from patients with benign diseases, gastric and CRCs were dropped onto the chips. Using a 632.8 nm laser excitation, measurements were taken and two peaks appeared to be prominent in the SERS spectra at 1350 cm^{-1} and 1570 cm^{-1}. Polynomial fitting was then used to determine SERS peak height for each of the samples. In order to validate that the SERS peak height and the concentration of the serum samples were correlated the measurements were repeated at different dilutions of the same serum sample. It was found that 10-fold dilutions were the saturating dilution for the NHC chips so 10 fold dilutions were used throughout the rest of the experiment. Finally the SERS peak height for both of the prominent peaks was compared between the three groups of samples and it was found that the SERS peak heights in the benign samples was indeed significantly lower than in the cancerous samples. This was calculated using the Pearson product-moment correlation coefficient and the non-parametric Spearman's rank correlation coefficient.

Another slightly different approach to using SERS for clinical use for CRC is using SERS as a characterisation/validation tool when developing other nanoscale devices such as the work done by da Paz *et al*[76] (2012). In this study SERS was used as a characterisation tool in the development of maghemite nanoparticles as a theragnostic device for CRC[76]. The SERS active nanoparticles used in this study were functionalised with Anti-CEA antigen in the hope that they can be used to detect primary and metastatic CRC, it was hoped by the authors that these nanoparticles could then be developed for a variety of applications including magnetic resonance imaging (MRI) enhancement and targeted drug delivery.

Limitations of Raman and SERS in clinical applications

Raman and SERS based tools have shown potential that they will have a place as either an alternative or an adjunct to current diagnostic methods. The development of SERS biomarker detection could also lead to its use in personalized medicine. However, there are still some limitations of Raman and SERS techniques that will need to be overcome before they are routinely used in a clinical setting. These include: (1) Many Raman studies involve costly equipment and expensive substrates, there will need to be investigations into cost reduction for large scale applications; (2) Raman and SERS studies that are carried out in a laboratory will require sample handling and storage, the effect of handling samples and storage techniques on the performance of Raman based tools will need to be quantified; (3) Thermal damage thresholds of *in vivo* tissue and *ex vivo* tissue samples from the colon and rectum will need to be established; (4) Many studies for clinical use different analytical techniques, and still require the skill of the user to determine the results of these techniques. User-friendly software for diagnostic analysis of the spectra will need to be developed and tested for multi-user reliability; (5) Inter equipment variability studies will need to be carried out, Raman equipment can often be susceptible to variability from external factors such as room temp, laser stability, *etc.*; and (6) SERS based techniques have been subject

to reproducibility issues, CRC is a heterogeneous disease so if immunoassay style tools are to be used then large scale studies with clinical samples will need to be carried out.

CONCLUSION

In the field of cancer detection Raman spectroscopy and SERS has gone through a period of rapid progress in the last decade. The use of Raman in clinical applications for CRC has previously been dominated by the discrimination of cancerous *vs* non-cancerous tissue with only a few studies on the use of Raman with biofluids for CRC detection. There are currently successful *in vivo* Raman tools for real-time use during endoscopy. These tools can be used to gain molecular information through Raman imaging and traditional spectroscopy. Therefore, they aid current endoscopic techniques by giving extra molecular information that could potentially be missed using traditional methods. However, Raman tools are still in general expensive to produce and require specialist knowledge in order to operate the machinery. Furthermore, thermal thresholds for the damage of GI tissue need to be properly established before these tools can be ready for use in a routine clinical setting. In future, national multi-site trials that include large patient numbers are needed to study the thermal threshold of tissues. Future research into the large-scale manufacture (and miniaturisation) of Raman tools needs to be carried out to investigate variability between sites and investigate the cost effectiveness of Raman tools compared to current technology.

In order to detect low concentration analytes SERS has started to become an alternative method to Raman. SERS offers enhanced signals and reduced fluorescence compared to Raman. Current research uses different techniques to gain a SERS response from samples. One of the limitations of SERS based techniques has been that the variations in the plasmon resonance of nanostructures that cause a SERS response are subject to large variability. Therefore, in mixing style SERS methods research into reducing the variability in SERS response even across a single sample will need to be investigated. Another method of gaining a SERS response is through a SERS based immunoassay; this has been successfully used to detect the current accepted biomarker for CRC CEA. The immunoassay design is based on reducing variability by controlling separation of the SERS substrates. SERS immunoassay has the potential to have multiplex detection of analytes in both tissue and biofluids. This could be one of the biggest areas of development if CRC research follows that of diseases such as nasopharyngeal cancer[77]. Furthermore, if SERS is successfully used to detect different concentrations of biomarkers then this opens up the possibility into research towards personalised medicine and detecting changes in the levels of biomarkers using less invasive methods than are currently available in the United Kingdom (*i.e.*, through blood based testing). There are currently other CRC detection tests in development that are more advanced than SERS such as mSept9 blood based testing. However, SERS based detection aims to have detection limits below the current available technology; therefore it offers the possibility of research into new biomarkers for CRC based on Raman or SERS spectral signals. Furthermore SERS and Raman based techniques still have the ability to be developed into techniques that are used in conjunction with other developing detection methods.

Both Raman and SERS techniques will also need further research into producing a universal method of background subtraction and analysis of data. Currently many research groups use different methods of data analysis that can be complex and still require clinicians to interpret results using spectral knowledge. In order for Raman and SERS based detection to be implemented into a clinical setting simple, user-friendly programs will need to be produced that remove the need for interpretation of spectra by a user. If the spectral analysis is automated then Raman and SERS techniques have the potential to become "observer-independent" tools.

Raman and SERS techniques are currently still in development with the aim to be in regular use in a clinical setting. If the technological limitations are overcome then the techniques have the potential to produce more specific, affordable detection and screening for CRC that can be routinely used in a clinical setting as an alternative or an adjunct to current methods.

The final limitation to Raman and SERS based techniques will be that of persuading clinicians that the new technology can replace existing techniques, it is possible that national based trails showing the robustness of Raman and SERS techniques will go some way to achieving this.

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REVIEW

Current adjuvant treatment modalities for gastric cancer: From history to the future

Leyla Kilic, Cetin Ordu, Ibrahim Yildiz, Fatma Sen, Serkan Keskin, Rumeysa Ciftci, Kezban Nur Pilanci

Leyla Kilic, Department of Medical Oncology, Acibadem University Hospital, Istanbul 34394, Turkey

Cetin Ordu, Department of Medical Oncology, Istanbul Bilim University, Istanbul 34394, Turkey

Ibrahim Yildiz, Fatma Sen, Serkan Keskin, Rumeysa Ciftci, Department of Medical Oncology, Institute of Oncology, Istanbul University, Istanbul 34394, Turkey

Kezban Nur Pilanci, Department of Medical Oncology, Haseki Training and Research Hospital, Istanbul 34394, Turkey

Author contributions: Kilic L and Ordu C contributed equally to this work as first authors; Kilic L, Ordu C and Yildiz I wrote the paper; Kilic L, Sen F, Keskin S, Pilanci KN performed the research; Ordu C, Yildiz I and Sen F designed the research; Ordu C, Yildiz I, Ciftci R, Pilanci KN analyzed the data.

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Correspondence to: Cetin Ordu, MD, Department of Medical Oncology, Istanbul Bilim University, Büyükdere Cad. No.120, Şişli, Istanbul 34394, Turkey. cetinordu@hotmail.com
Telephone: +90-532-2276179
Fax: +90-212-2889812

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Abstract

The discrepancy between the surgical technique and the type of adjuvant chemotherapy used in clinical trials and patient outcomes in terms of overall survival rates has led to the generation of different adjuvant treatment protocols in distinct parts of the world. The adjuvant treatment recommendation is generally chemoradiotherapy in the United States, perioperative chemotherapy in the United Kingdom and parts of Europe, and chemotherapy in Asia. These options mainly rely on the United States Intergroup-0116, United Kingdom British Medical Research Council Adjuvant Gastric Infusional Chemotherapy, and the Asian Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer and Capecitabine and Oxaliplatin Adjuvant Study in Stomach Cancer trials. However, the benefits were evident for only certain patients, which were not very homogeneous regarding the type of surgery, chemotherapy regimens, and stage of disease. Whether the dissimilarities in survival are attributable to surgical technique or intrinsic biological differences is a subject of debate. Regardless of the extent of surgery, multimodal therapy may offer modest survival advantage at least for diseases with lymph node involvement. Moreover, in the era of individualized treatment for most of the other cancer types, identification of special subgroups comprising those who will derive more or no benefit from adjuvant therapy merits further investigation. The aim of this review is to reveal the historical evolution and future reflections of adjuvant treatment modalities for resected gastric cancer patients.

Key words: Adjuvant chemoradiotherapy; Biomarker; Gastric cancer; Lymph nodes

Core tip: Despite extensive surgery, gastric cancer will likely recur for most patients. Fortunately, additional treatment modalities either in the perioperative or post-operative setting provide varying degrees of survival

advantage. Although there is considerable data regarding adjuvant chemotherapy and chemoradiotherapy since the Intergroup-0116 study, there is still no established uniform treatment protocol depending on the type of surgery, histological subgroup, or extent of disease. The present review is aimed at identifying the advances in treatment strategies and discussing the pros and cons of each strategy.

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INTRODUCTION

Gastric cancer is the fifth most common cancer in the world, with 952000 new cases being diagnosed in 2012[1]. The cornerstone for treatment of gastric cancer is surgical resection with lymph node dissection (LND). An extended lymph node resection, which is commonly defined as a D2 resection for gastric cancer, includes the removal of the whole stomach, the greater and lesser omentum, and the N1 and N2 (groups 1-11) lymph nodes. For tumours located in the proximal stomach, resection of the spleen and the tail of the pancreas may be necessary for removing groups 10 and 11 lymph nodes. While a D2 lymph node dissection is considered a standard surgical procedure for resectable gastric cancer in Japan and Korea, the necessity of a D2 dissection still remains a subject of controversy in Western countries. The overall 5-year survival rates in the United States is around 10% to 40%; however, in Japan and South Korea, it is reported to be 50% or higher[2-4]. It is unclear whether removing additional lymph nodes during the operation contributes to a difference in survival. Additional information on lymph nodes may provide more accurate staging, which is currently only available for patients that undergo a D2 dissection. Other factors such as early diagnosis, case selection, surgical skill, and post-operative care may also contribute to this observed difference in survival.

Despite difficulties with surgical techniques, data from the National Cancer Data Base in the United States points at a 10-year survival rate of 65% for patients with resected stage IA disease and 3%-42% for those with more advanced disease[5]. Thus, the high rate of both locoregional and distant relapse, even after complete resection, makes adjuvant treatment mandatory for patients with stomach cancer.

The timing and sequence of adjuvant/neoadjuvant strategies and combination therapies have been questioned in numerous phase Ⅱ and phase Ⅲ trials. The landmark Intergroup (SWOG 9008/INT-0116) trial demonstrated a survival benefit for resected stage IB-Ⅳ, M0 gastric cancer patients following adjuvant chemoradiotherapy[6]. However, the extent of surgery and the chemotherapy (CT) regimen, which was associated with high rates of toxicity, was seriously critiqued in this study. Although an extensive (D2) LND was recommended, only 10% of the patients had undergone a D2 dissection. The relative inadequacy of locoregional control with limited LND was supposed to be compensated with adjuvant radiotherapy.

Although there is large amount of data from randomized, controlled trials (RCT) and recommendations from several guidelines, recent analysis from the National Cancer Data Base have revealed that real-life practices somewhat diverge from the evidence-based results[7]. Trends in American cancer centres have shown that out of stage Ⅲ patients who received surgery at community hospitals, less than 50% also received adjuvant chemoradiotherapy in 2009. However, the large number of patients involved in the RCTs constitute a heterogeneous population where the benefit from adjuvant therapy may be difficult to interpret for some specific subgroups of patients. Moreover, there is still no phase Ⅲ data supporting the tailoring of treatment according to stage of disease after surgery, unlike colon and breast cancers, since each stage may benefit from adjuvant therapy to a varying degree. This review will focus on the evidences of adjuvant treatment strategies dependent on the recent RCTs and future directions for optimal approach.

ADJUVANT CHEMOTHERAPY REGIMENS

Surgical resection is the only hope for curative treatment in early stages of gastric cancer. However, only 40% of the patients with gastric cancer will remain disease free after complete resection of their tumour. Therefore, adjuvant and neoadjuvant treatment modalities are crucial for establishing better prognosis for gastric cancer patients. Extensive studies were conducted to determine the efficacy of adjuvant chemotherapy for gastric cancer. Some previous phase Ⅲ randomized trials did not demonstrate absolute benefit for adjuvant chemotherapy. However, these studies usually did not enrol large datasets and generally included early stage patients[8-10]. The Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer (ACTS-GC) study was a pioneer phase Ⅲ study showing a significant survival benefit following adjuvant chemotherapy after D2 LND for gastric cancer patients. The trial documented that one year adjuvant chemotherapy with an oral fluoropyrimidine, S-1 provided a clear survival benefit for stage Ⅱ and Ⅲ gastric cancer patients after D2 LND[3]. Since the publication of the results of the ACTS-GC trial, chemoradiation was excluded from adjuvant treatment modalities for D2 resected gastric cancer in Japan, but this was not the case in Western countries. However, the results of the ACTS-GC study conflicted with a similar large scaled phase Ⅲ Japanese trial that utilized mitomycin C, fluorouracil (FU), and oral UFT (a combination of tegafur, a prodrug of 5-FU and uracil treatment) as adjuvant chemotherapy[11]. The investigators considered that this

result was due to high proportion of pT1 gastric cancer patients included in the trial for which surgery alone may yield a good prognosis, and there seemed to be no requirement for adjuvant therapy. Consequently, further trials usually did not include early stage patients (*i.e.,* ≤ stage Ib).

Apart from the Asian studies, most of the trials performed with adjuvant chemotherapy have not demonstrated a significant survival benefit[12-14]. However, the results of the meta-analysis by the Global Advanced/Adjuvant Stomach Tumour Research International Collaboration (GASTRIC) group with an extended follow-up time have revealed a modest but statistically significant survival advantage with adjuvant chemotherapy after curative resection of gastric cancer[15]. There was an absolute improvement in overall survival (OS) of 6% after 5 years that was maintained at 10 years. The treatment benefit was sustained in the majority of the investigated groups of FU-based regimens, with reductions in the risk of death between 20% and 40%. This meta-analysis pointed out that adjuvant FU-based chemotherapy is associated with improved OS, and combination chemotherapy could be recommended for patients who have not received treatment in the perioperative setting.

S-1, an orally active FU analogue, is a combination of tegafur (a prodrug of 5-FU), gimeracil (an inhibitor of dihydropyrimidine dehydrogenase), and oteracil (inhibitor of the phosphorylation of FU in the gastrointestinal tract). Pharmacokinetics studies have shown that the absorption of FU derived from S-1 is not affected by gastrectomy[16]. The response rates with S-1 alone were higher than 40% in two phase Ⅱ trials among patients with advanced gastric cancer[17,18]. Similarly, S1 demonstrated a clear survival benefit in the ACTS-GC study. After 3 years of median follow-up, the OS in the S-1 group was 33% higher than the surgery-only group[3]. Grade 3 or grade 4 adverse events occurred in less than 5% of patients in the S-1 group. The OS rate was 80.5% in the S-1 group and 70.1% in the surgery-only group at 3 years. Thus, S-1 was approved as an effective option for adjuvant chemotherapy for patients with resected gastric cancer.

Recently Zhang *et al*[19] have published the analysis of 31 RCTs, which included 7120 gastric cancer patients. There was no significant difference in terms of overall mortality among the four chemotherapy regimens including FU + mitomycin (MMC) + adriamycin, FU + MMC (FM), Tegafur and MMC. The evidence for the FM regimen and MMC regimen was not strong enough. According to this meta-analysis, Tegafur was recommended as the first-line adjuvant chemotherapy regimen for patients after complete resection. However, RCTs published after 2000 have consisted of primarily combinations of cisplatin and FU. Collectively, S1 or 5-FU-cisplatin combination regimens in neo-adjuvant, adjuvant, and perioperative settings have yielded a favourable impact on survival[20-32]. Moreover, chemotherapy seems to provide prolongation of survival for patients with mostly node-positive and T3-T4 disease (Table 1).

The combination of adjuvant with neo-adjuvant chemotherapy has proven its value in two randomized trials. As the pioneer study of perioperative chemotherapy for gastric cancer, the British Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) trial demonstrated a significant downstaging of the primary tumour and a 10% higher resectability rate with a survival benefit of 13% at 5 years[31]. The primary goals of the ECF perioperative CT were to increase the likelihood of R0 resection while downstaging the tumour, predicting tumour sensitivity to chemotherapy, improving obstructive symptoms, and eliminating micrometastases. One of the major limitations of the MAGIC trial was that only 42% of patients in the chemotherapy group were able to receive all protocol treatment; 34% of patients who completed preoperative chemotherapy and surgery could not be administered postoperative chemotherapy, possibly due to postoperative complications, early disease progression, or the patients' will. Nevertheless, patients in the perioperative chemotherapy section had a survival advantage when compared with those who underwent surgery alone (5 years OS rate for CT group *vs* surgery-alone; 36% *vs* 23%, respectively).

New questions have arisen regarding the optimal adjuvant therapy following the increased acceptance of D2 gastrectomy. The primary goal of design for Capecitabine and Oxaliplatin Adjuvant Study in Stomach Cancer (CLASSIC) study was to answer these questions. The investigators aimed to evaluate the effect of adjuvant capecitabine plus oxaliplatin after D2 gastrectomy[25]. The CLASSIC study reported a 44% improvement in disease-free survival (DFS) for patients randomly assigned to postoperative capecitabine and oxaliplatin (XELOX) when compared with observation. Subgroup analysis confirmed the beneficial effect of adjuvant capecitabine and oxaliplatin for all disease stages (Ⅱ, ⅢA or ⅢB), and the extent of nodal involvement correlated with substantially more benefit (N2 > N1 > N0) from adjuvant CT. Three-year DFS was defined as the primary endpoint because the majority of the recurrences occur within 3 years of surgery according to the preliminary data from the GASTRIC group. Although not formally validated as a surrogate measure yet, 3-year DFS is strongly correlated with 5-year OS, which is the reference point for judging effectiveness of adjuvant therapy in gastric cancer[15]. Additionally, after a median follow-up of 62.4 mo, the updated results supported the interim analysis findings; the estimated 5-year DFS was 68% in the adjuvant capecitabine and oxaliplatin group *vs* 53% in the observation alone group[33]. The OS data from this study are not yet known; however, the data suggest an improvement in OS with capecitabine and oxaliplatin compared with surgery alone (78% *vs* 69%).

Whether the results of CLASSIC trial could be adapted to geographical regions where management practices differ is unclear. The CLASSIC trial had considerably better survival outcomes when compared with Western counterparts; the 3-year OS rate in the surgery-only group was 78% in the CLASSIC trial, while it was only 30%-40% in the United States Intergroup-0116 and

Table 1 Randomized trials of chemotherapy for resected gastric cancer, published after 2000s

Ref.	Regimen	LN (+) % (chemo)	T3-T4 % (chemo)	No. of patients	D2 % (chemo)	% 5-yr survival	*P* value
Neoadjuvant							
Schuhmacher *et al*[20]	5-FU, LV, cisplatin	94.4	100	72	95.7	72.7	0.2
	Surgery alone			72		69.9	
Adjuvant							
Bajetta *et al*[21]	EAP, 5-FU + LV	91	51	137	73	52	0.87
	Surgery alone			137		48	
Chipponi *et al*[22]	5-FU, cisplatin	80	75	101		39.0	NS
	Surgery alone			104		38.7	
Bouché *et al*[23]	5-FU, cisplatin	80.3	77.9	127	55.9	46.6	0.22
	Surgery alone			133		41.9	
Sasako *et al*[24]	S-1	90.4	45.1	529	100	71.7	
	Surgery alone			530		61.1	
Bang *et al*[25]	Capecitabine, oxaliplatin	91	45	520	100	83	0.493
	Surgery alone			515		78	
Kang *et al*[26]	MMC + 5'FDR (MF') *vs*	90	43	424	100	66.5	0.33
	MF' + CDDP			431		65	
Di Costanzo *et al*[27]	PELF	83.8	49.2	130	55	47.6	0.41
	Surgery alone			128		48.7	
De Vita *et al*[28]	ELFE	72	80	112	79	48	0.610
	Surgery alone			113		43.5	
Nitti *et al*[29]	FAMTX or FEMTX	82	61	194	88	43	0.86
	Surgery alone			203			
Neri *et al*[30]	EPI, LV, 5-FU	100	84	69		30 m's (median)	< 0.01
	Surgery alone			68		18	
Perioperative							
Cunningham *et al*[31]	EPI, 5-FU, cisplatin	71	56	250	28	36.3	0.009
	Surgery alone			253		23.0	
Ychou *et al*[32]	5-FU, cisplatin	67	58	113	100	38	0.02
	Surgery alone			111		24	

5-FU: 5-fluorouracil; EAP: Etoposide, adriamycin, cisplatin; LV: Leucovorin; MMC: Mitomycin C; 5' FDR: Doksifluridine; EPI: Epirubicine; FAMTX: Fluorouracil, adriamycin; methtrexate; FEMTX: Fluorouracil, epirubicin; methtrexate; PELF: Cisplatin, epirubicin, leucovorin, 5- fluorouracil; ELFE: Epirubicin, leucovorin, 5-fluorouracil, etoposide; CDDP: Cisplatin; LN: Lymph node.

United Kingdom MAGIC populations. Although patients included in the CLASSIC trial had fewer T3 and T4 lesions (44% in CLASSIC *vs* 68% in Intergroup-0116 *vs* 64% in MAGIC), node-positive disease was more frequent (90% *vs* 85% *vs* 72%). The differences in survival rates are supposed to be not only due to prognostic differences but also due to intrinsic biological disparities and consistent use of D2 surgery. Since D2 gastrectomy is also a standard of care in Western countries currently, the findings of this study could be remarkable and generalized to the other regions where D2 surgery is performed by experienced surgeons.

ADJUVANT CHEMORADIOTHERAPY

After surgery with curative intent, local or regional recurrence in the gastric or tumour bed, the anastomosis, or regional lymph nodes occurs in 40% to 65% of patients[34]. According to the preoperative analysis of the University of Minnesota, locoregional failure was the only evidence of relapse in 29% of patients and as any component of relapse in 88% of patients. Locoregional recurrences occurred in three major locations: (1) Gastric bed (organs and structures in proximity to the primary tumour); (2) regional nodes; and (3) gastric remnant, anastomoses, and duodenal stump[35]. Autopsies report even higher locoregional failure rates reaching up to 80%-93%[36]. Thus, radiotherapy is supposed to be an essential adjunct of postoperative treatment in gastric cancer patients.

Two randomized trials evaluating the benefit of adjuvant radiotherapy (RT) alone after resection for gastric cancer revealed conflicting results. The first trial by the British Stomach Cancer Group included 436 patients who were randomized to undergo surgery alone or surgery followed by RT or chemotherapy with mitomycin, doxorubicin, and fluorouracil[37]. However, more than one third of the patients had gross or microscopic residual disease following surgery. At the 5-year follow-up, there was no additional survival benefit for adjuvant RT or chemotherapy compared with surgery alone. However, there was a significant reduction in locoregional recurrence with the addition of RT to surgery. The second trial by Zhang *et al*[38] randomized 370 patients to preoperative RT or surgery alone. Survival and resection rates were significantly improved with preoperative RT compared with surgery alone (30% *vs* 20%; 89.5% *vs* 79%, respectively). However, there was not a significant reduction in distant recurrence rates (24.3% *vs* 24.7%). Because only cardiac lesions were included in the trial, it is not clear whether these results can be adapted to distal lesions.

The landmark trial regarding combined modality adjuvant treatment for gastric cancer has been the

Intergroup 0116 study[6]. A total of 556 patients with resected carcinoma of the stomach or gastroesophageal junction (stage IB through Ⅳ, M0) disease were randomized to surgery alone or surgery plus postoperative chemoradiotherapy. The median OS in the surgery only group was 27 mo, as compared with 36 mo in the chemoradiotherapy group (P = 0.005). An important issue regarding the surgical procedure was the extent of surgery in this trial. Although the recommendation was an extensive D2 LND, only 10% of the patients underwent a D2 dissection, 36% had a D1 dissection, and more than half had a D0 lymphadenectomy (not all of the N1 nodes were resected). This situation raised the question of whether chemoradiation was compensatory for inadequate surgery. Thus, in high-volume centres where D2 LND is routinely performed, omitting adjuvant radiotherapy has been considered due to high morbidity rates and poor tolerance. However, an observational study including patients with D2 LND has demonstrated that chemoradiotherapy as an adjunct to surgery could be tolerable with acceptable toxicity and good tumour control[39]. The patients had received a postoperative chemoradiotherapy protocol similar to the Intergroup trial or surgery without further adjuvant treatment. The median duration of OS was significantly longer in the chemoradiotherapy (CRT) group than in the comparison group (95.3 mo *vs* 62.6 mo). However, these data rely on nonrandomized observation studies with suitable controls or unplanned subgroup analysis.

Updated analysis of the Intergroup trial with longer follow-up has supported the persistent benefit of adjuvant CRT[40]. The OS and recurrence-free survival data demonstrated continued strong benefit from postoperative radiochemotherapy. Hazard ratios were virtually unchanged since the original report. Moreover, two meta-analyses comparing the efficacy of adjuvant CRT *vs* CT after R0 resection have confirmed the superiority of the combined modality in terms of disease-free survival[41,42]. However, there was no OS advantage with the addition of radiotherapy in both analyses.

OPTIMAL CHEMOTHERAPY REGIMEN DURING RADIOTHERAPY

One of the most criticized aspects of the Intergroup trial was the toxicity profile of the FU/LV regimen. Therefore, other investigators have sought alternative postoperative chemoradiation regimens. In a pilot study by Lee *et al*[43] patients with stage Ⅲ-Ⅳ (M0) gastric cancer who had undergone extensive D2 LND were administered postoperative chemoradiation with fluorouracil and cisplatin before and after capecitabine and concurrent RT. A total dose of 4500 cGy in 25 fractions over five weeks was delivered to the target volume similar to the INT-0116 trial. This study demonstrated a 3-year disease free and OS of 82.7% and 83.4%, respectively, with the use of adjuvant chemoradiotherapy. Leong *et al*[44] reported that postoperative chemotherapy with epirubicin, cisplatin, and 5-FU (ECF) before and after concurrent chemoradiation with infusional fluorouracil was tolerable and efficient. A similar regimen with ECF before and after radiation with infusional fluorouracil has been compared with the INT-0116 regimen in a randomized phase Ⅲ trial (CALGB 80101)[45]. Although the ECF regimen had a more favourable toxicity profile compared with bolus fluorouracil and leucovorin, there was no significant improvement in survival.

Alternative regimens for concomitant and adjuvant treatment have also been experienced. The efficacy of paclitaxel - cisplatin and 5-FU regimen against oesophageal and gastroesophageal junction adenocarcinomas has been demonstrated previously[46]. Results of a phase Ⅰ trial conducted among patients with locally advanced gastric cancer using weekly cisplatin and RT with paclitaxel as a 96-h continuous infusion were also promising[47]. Another trial from MD Anderson included patients with gastric cancer who received two cycles of induction chemotherapy with infusional 5-FU, cisplatin on days 1 to 5, and paclitaxel over 24 h on day 1 of each 28-d cycle[48]. During the 5-wk course of RT, infusional 5-FU and paclitaxel were administered weekly. Complete and partial response rates were 22% and 15%, respectively, which were quite promising. In the light of these findings, a phase Ⅱ trial (RTOG-0114) was designed to integrate paclitaxel and cisplatin with or without 5-FU concomitantly through radiotherapy. However, the paclitaxel, cisplatin, and 5-FU (PCF) arm were closed due to the high gastrointestinal toxicity rates, which were significantly worse than INT0116 results[49]. For the paclitaxel and cisplatin (PC) arm, the 2-year DFS was 52% (95%CI: 36%-68%). Although the PC arm was tolerable, the DFS failed to exceed the predefined lower bound of DFS at 2 years. Thus, this regimen could not be recommended as an adjuvant modality for future randomized phase Ⅲ studies. These trials suggested that intensification of adjuvant chemotherapy or chemoradiation regimens may not be as effective as expected. Table 2 summarizes the major trials evaluating adjuvant CRT.

Whether an intensified CRT regimen prolonged survival after D2 dissection was another subject of debate. The ARTIST trial was designed to answer this question; comparing six cycles of capecitabine and cisplatin (XP) chemotherapy with two cycles of XP followed by concurrent capecitabine and RT followed by two additional cycles of XP after D2 dissection[4]. However, the study failed to demonstrate a significant DFS benefit with the addition of radiotherapy to XP (3-year DFS rates 78.2 *vs* 74.2% for CRT and CT arms, respectively). Treatment was completed as planned by 75.4% of patients in the chemotherapy arm and 81.7% in the CRT arm. The updated analysis with longer follow-up did not reveal DFS or OS benefit either[50]. However, the subgroup of patients with pathologic lymph node metastasis in the CRT arm had superior DFS when compared with those who received CT alone (3-year DFS; 76% *vs* 72%). Besides,

Table 2 Features of major adjuvant chemoradiotherapy trials for gastric cancer

Ref.	CT Regimen without RT/with RT	*n* (total)	D2 rates	G3-G4 toxicity (hem/GI)	Completeness of treatment	RT technique
Macdonald *et al*[6]	Bolus 5-FU + LV/bolus 5-FU + LV	556	10%	54%/33%	64%	2D
Lee *et al*[43]	FP/capecitabine	31	100%	50.2%/12.8%	74.20%	2D
Zhu *et al*[57]	Bolus 5-FU + LV/bolus 5-FU + LV	380	100%	5.9%/7.5%	NA	IMRT
Leong *et al*[44]	ECF/inf 5-FU	54	NA	66%/28%	NA	3D
Schwartz *et al*[49]	PC (PCF arm closed)/PC	78	NA	24%/33% (for PC arm)	NA	3D
Lee *et al*[4]	XP/capecitabine	458	100%	48.4%/19%	81.7%	3D

CT: Chemotherapy; RT: Radiotherapy; D2: D2 lymph node dissection; G3-G4: Grade 3-grade 4; hem: Hematologic; GI: Gastrointestinal; NA: Not available; 5-FU: 5-fluorouracil; LV: Leucovorin; inf: Infusional; FP: 5-FU, cisplatin; ECF: Epirubicin, cisplatin, 5-FU; PC: Paclitaxel, cisplatin; PCF: Paclitaxel, cisplatin, 5-FU; XP: Capecitabine, cisplatin; 2D: Two-dimensional; 3D: Three-dimensional; IMRT: Intensity modulated radiotherapy.

intestinal-type gastric cancer derived more benefit from CRT (3-year DFS rates were 83% and 94% in the CT and CRT arms, respectively).

The most commonly encountered nonhaematologic grade 3 to 4 side effects were stomatitis, hand and foot syndrome, diarrhoea, and vomiting, each of which occurred in 1% to 12% of patients in both arms. The rate of grade 3 and 4 neutropenia was 39% in the CT arm and 48% in the CRT arm; however, the rate of febrile neutropenia was quite low in both arms (< 1%), suggesting that postoperative treatment with cisplatin and capecitabine and is tolerable following a D2 LND. In conclusion, capecitabine at a dose of 1650 mg/m^2 per day with RT was well tolerated.

In the light of the studies mentioned above, combination regimens other than 5-FU/LV are still under investigation for gastric cancer. The NCCN guidelines, however, do not recommend the standard bolus 5FU/LV regimen utilized in the INT0116 trial. Relying on the data from gastric cancer trials, such as the ARTIST trial and colorectal cancer studies, capecitabine or infusional 5-FU is recommended concomitantly with RT by the NCCN due to their better toxicity profile and tolerability[51].

RADIOTHERAPY TECHNIQUE

Since the publication of the INT0116 results, the radiotherapy technique and planning of the target volume has changed over time. Patients involved in this trial received 45 Gy of radiation in 25 fractions to the surgical bed, regional lymph nodes, and preoperative tumour volume. The regional lymph nodes included perigastric, splenic, hepatoduodenal, pancreatoduodenal, celiac, and local paraaortic lymph nodes based on patterns of failure after a D0/D1 dissection. In the Intergroup-0116 trial, two-dimensional (2D) radiation therapy was utilized and the CRT arm was associated with high rates of toxicity, with nearly three-quarters of patients experiencing grade 3/4 toxicities. Only 64% of patients in the CRT arm completed the planned treatment program, and 17% discontinued treatment due to toxicity. However, treatment-related mortality was low (1% on the chemoradiation arm *vs* 0% on the surgery alone arm). In addition, overall chemoradiation appeared tolerable.

Fortunately, technology has improved over time to allow conformal radiation therapy, sparing normal tissues and allowing dose escalation. Three-dimensional (3D) conformal radiotherapy reduces the damage to normal tissues to some extent and is considerably superior to 2D radiation[52]. Currently, modern 3D RT techniques are applied for the resected gastric cancer patients at most of the oncology centres in the world. 3D planning enables exact description of the target volume and organs at risk by visualization of anatomic changes in the internal organs after surgery[53].

Whether or not to change the RT target volumes for patients undergoing D2 dissection is another subject of debate. The findings of the study by Chang *et al*[54] have revealed that the most prevalent sites of regional recurrence after D2 dissection were the lymph nodes around the superior mesenteric vessels, the abdominal aorta from the upper margin of celiac trunk to the lower margin of aortic bifurcation, and the hepatoduodenal lymph nodes, which were primarily in the nodal basin outside the D2 dissection field. Consistent with these findings, the RT target volume in the ARTIST trial did not involve lymph nodes in the perigastric region and splenic hilum[4]. The investigators have noted higher locoregional relapse rates in the CT arm (13% *vs* 7%, $P = 0.0033$) which supports the addition of CRT even in the presence of D2 dissection with the modified RT target volume.

Intensity modulated radiotherapy (IMRT) is a more sophisticated radiotherapy technique, with capability of delivering high doses of radiation to a targeted area with high geometrical accuracy. According to the recent studies, IMRT for gastric cancer is dosimetrically superior to conventional therapy, because IMRT is able to decrease the radiation dose to organs at risk, especially the spinal cord and kidney, while providing the intended radiation dose to the target areas[55,56]. The most recent phase Ⅲ trial comparing concomitant CT with IMRT and chemotherapy alone investigated the role of IMRT among gastric cancer patients with D2 LND[57]. The IMRT plus CT arm was tolerable with a significant improvement in DFS (5-year DFS, 45% *vs* 36%); however, the results of this trial could not point at an OS benefit like the previous comparative studies (5-year OS, 24% *vs* 27%, $P > 0.05$). According to some investigators, IMRT appears to provide only limited advantages when compared with sophisticated 3D conformal RT planning[58]. Moreover, the risk of a second cancer induced by radiation is reported

to increase in some patients[59,60]. Whether 3D conformal RT or IMRT provides better protection of organs at risk remains controversial.

SELECTING PATIENTS FOR ADJUVANT CHEMORADIOTHERAPY

Stage

Although the patients involved in the INT0116 trial were stage IB-Ⅳ (M0), the majority had advanced disease, whereas up to 60% of patients in the ARTIST trial were stage Ⅰ/Ⅱ. Furthermore, in the subgroup analysis of the ARTIST trial, improved DFS ($P < 0.05$) was observed in stage Ⅲ and Ⅳ patients in the CRT group. The proportion of stage Ⅲ/Ⅳ (M0) patients enrolled in the study by Zhu *et al*[57] was 71%, which demonstrated a DFS benefit for CT with IMRT after D2 dissection. The subset (node-positive) analyses of the ARTIST trial and the DFS advantage for stage Ⅲ and Ⅳ (M0) patients in the Chinese trial supported that the use of adjuvant CRT for the whole stage IB to stage Ⅳ (M0) population may be overtreatment. Similarly, adjuvant CT alone may be inadequate for resected stage Ⅲ-Ⅳ patients. Subgroup analysis of 5-year OS in the ACTS-GC trial from Japan showed an insufficient survival benefit of S1 for N3a and N3b stages (HR = 0.77, 95%CI: 0.53-1.13 and HR = 0.92, 95%CI: 0.47-1.79, respectively). The results indicated the necessity of adjuvant RT in these patients who were at high risk for locoregional relapse. Accordingly, in our study, which included D2 dissected pN3(M0) gastric cancer patients, the addition of RT to CT did not provide a statistically significant improvement in DFS or OS, but there was an evident difference between the CT and CRT arms numerically (median DFS 12.5 and 15.2 mo; median OS, 26.8 mo *vs* 34.2 mo for CT and CRT arms, respectively)[61]. Another retrospective study from China including stage Ⅲ gastric cancer patients with D2 dissection showed OS and DFS advantage for stage ⅢC patients undergoing adjuvant CRT compared with CT[62]. These studies indicate that patients with relatively advanced disease stages (Ⅲ or Ⅳ) would benefit the most from adjuvant CRT.

The presence of lymphovascular invasion (LVI) or perineural invasion (PNI) have been demonstrated as significant prognostic factors for both OS and DFS among patients undergoing adjuvant treatment[63]; however, thus far, there have not been any randomized trial data evaluating the administration of CT or CRT depending on the stage or presence of LVI or PNI, unlike for breast and colon cancers. However, the ARTIST Ⅱ trial is on track to evaluate the efficacy of all available adjuvant treatment modalities after D2 dissection for node positive patients (clinicaltrials.gov NCT01761461); chemotherapy with S-1 for 1 year *vs* chemoradiotherapy involving two cycles of SOX followed by S-1/radiotherapy and then four additional cycles of (SOX) *vs* combination chemotherapy with S-1 and oxaliplatin (SOX) for 6 mo. Patients were stratified according to stage, type of surgery, and the Lauren classification.

Another phase Ⅲ study is currently recruiting stage IB gastric cancer patients for evaluating adjuvant capecitabine *vs* observation (clinicaltrials.gov NCT01917552). The results of these trials are expected to answer the question regarding tailoring treatment to disease stages.

Histology and biomarkers

The main carcinogenic event for the evolution of diffuse type of gastric cancer is loss of expression of E-cadherin, a key cell surface protein for establishing intercellular connections. Biallelic inactivation of the gene encoding E-cadherin, CDH1, can occur through germline or somatic mutation, allelic imbalance events (*e.g.,* loss of heterozygosity), or epigenetic silencing of gene transcription. Diffuse type cancers are highly metastatic and characterized by rapid disease progression and a poorer prognosis than intestinal cancers[64]. Thus far, there has been no adjuvant therapy trial designed according to histological subtype. However, exploratory subgroup analysis of randomized trials point at varying degrees of benefit according to histologic subtype. The investigators of the INT0116 study reported their observation of a reduced treatment benefit in patients with diffuse histology in their updated analysis[40]. Similarly, the patients with intestinal type gastric cancer were found to be more prone to benefit from CRT than those with diffuse type in subgroup analyses of the ARTIST trial[50]. Patients with intestinal type histology showed a significant improvement in DFS in the CRT arm compared with the CT arm (94% *vs* 83%, $P = 0.01$, respectively). Whether or not this is a random observation of an unplanned subset analysis or reflective of the biologic variations is unknown, but if chemoradiotherapy is less effective in diffuse gastric cancer, future clinical trials may consider different adjuvant strategies based on histological subtype. A phase Ⅱ/Ⅲ study is currently recruiting patients with resectable signet-ring cell gastric carcinoma to perioperative treatment similar to MAGIC trial or surgery followed by six cycles of ECF (clinicaltrials.gov NCT01717924). This study may help determining the efficacy of intense CT with cisplatin for diffuse type gastric cancer cases.

In addition to morphologic appearance and clinical behaviour, the two distinct types of gastric adenocarcinoma differ with respect to their pathogenesis and genetic profiles[65]. For the intestinal subtype, there is meticulous evidence for the role of *Helicobacter pylori* in the initiation of the events that lead from chronic active gastritis to atrophic gastritis, intestinal metaplasia, dysplasia, and finally adenocarcinoma. Many gene changes have been described in various stages of the preneoplastic/neoplastic cascade, but the alterations do not generally follow a sequential arrangement. Some changes are seen in early preneoplastic lesions but are not present in more advanced lesions. Therefore, it is not easy to develop an appropriate target for treatment.

Approximately 50% of intestinal-type gastric cancers have alterations in tumour suppressor genes, including

TP53, TP73, *APC*, *TFF*, *DCC*, and *FHIT*[66]. In addition, epigenetic alterations, such as DNA methylation of gene promoters, can silence the expression of certain genes, including *CDH1* (the E-cadherin gene), in not only diffuse type but also in intestinal-type cancers[67,68]. Unlike the complex molecular pathway for intestinal type, diffuse carcinomas display a discriminative molecular abnormality: Defective intercellular adhesions through the loss of expression of the cell adhesion protein E-cadherin as mentioned below. Although this knowledge has not resulted in a specific targeted therapy for diffuse gastric cancer yet, the recognition of germline *CDH1* mutations in families helps identify high-risk individuals and encourage them to receive prophylactic gastrectomy.

The struggle to identify specific biomarker for predicting a treatment benefit has not resulted in success in the adjuvant setting so far. In the ARTIST trial, the different status of the *EGFR*, *HER-2, MET, MLH1,* and *CDH1* genes were considered; however, differences in the expression of these genes between the CRT and CT groups had no effect on DFS[50]. Inhibition of HER-2 overexpression *via* trastuzumab in metastatic disease has revealed a median of 2.7 in OS benefit in the ToGA trial, and currently this strategy is being evaluated in the adjuvant setting (clinicaltrials.gov NCT01130337, NCT01748773). Previously, the amplification of mesenchymal-epithelial transition (MET) receptor has been linked to poorer clinical outcome in patients with gastric cancer[69]. There are some conflicting case reports on attempts to target MET in patients with gastric cancer[69,70]. However, it seems feasible to wait until the results of the trial, which is testing a MET antibody in the metastatic setting, are reported (clinicaltrials.gov NCT01662869).

CONCLUSION

Currently, there is no doubt that adjuvant chemotherapy or chemoradiotherapy after resection of gastric cancer offers survival benefits. The major challenge for the clinicians is how and where to place the additional treatment modality (*i.e.*, CT or CRT; adjuvant or perioperative setting). The selection of the appropriate patient who will provide more or no benefit from therapy further complicates the situation. Obviously, there is lack of data to compare perioperative CT *vs* adjuvant CRT. However, the evidence for adjuvant CT with XELOX or S1 after D2 dissection is satisfactory. Although the evolution of the RT technique since the Intergroup study promises better tolerability, the addition of CRT after D2 dissection merits further investigation in the light of the findings from the ARTIST trial. Instead of the bolus 5-FU regimen or 5-FU combinations, infusional 5-FU or capecitabine concomitantly with RT may be preferred due to the improved toxicity profile. Nevertheless, the struggle to individualize treatment strategies for a robust combat with the resistant subgroups, such as the diffuse type of gastric cancer, should continue until optimal targets for therapy are defined.

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Multitarget stool DNA for colorectal cancer screening: A review and commentary on the United States Preventive Services Draft Guidelines

Barry M Berger, Bernard Levin, Robert J Hilsden

Barry M Berger, Medical Affairs, Exact Sciences Corporation, Madison, WI 53719, United States

Bernard Levin, Scientific Advisory Board, Exact Sciences, New York, NY 10025, United States

Robert J Hilsden, Departments of Medicine and Community Health Sciences, University of Calgary, Calgary AB T2N 4N1, Canada

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Correspondence to: Barry M Berger, MD, Chief Medical Officer, Medical Affairs, Exact Sciences Corporation, 5801 Research Park Blvd, Madison, WI 53719, United States. bberger@exactsciences.com
Telephone: +1-608-5358553

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Abstract

Multitarget stool DNA (mt-sDNA) testing was approved for average risk colorectal cancer (CRC) screening by the United States Food and Drug Administration and thereafter reimbursed for use by the Medicare program (2014). The United States Preventive Services Task Force (USPSTF) October 2015 draft recommendation for CRC screening included mt-sDNA as an "alternative" screening test that "may be useful in select clinical circumstances", despite its very high sensitivity for early stage CRC. The evidence supporting mt-sDNA for routine screening use is robust. The clinical efficacy of mt-sDNA as measured by sensitivity, specificity, life-years gained (LYG), and CRC deaths averted is similar to or exceeds that of the other more specifically recommended screening options included in the draft document, especially those requiring annual testing adherence. In a population with primarily irregular screening participation, tests with the highest point sensitivity and reasonable specificity are more likely to favorably impact CRC related morbidity and mortality than those depending on annual adherence. This paper reviews the evidence supporting mt-sDNA for routine screening and demonstrates, using USPSTF's modeling data, that mt-sDNA at three-year intervals provides significant clinical net benefits and fewer complications per LYG than annual fecal immunochemical testing, high sensitivity guaiac based fecal occult blood testing and 10-year colonoscopy screening.

Key words: Colorectal cancer screening; Multitarget stool DNA; Stool DNA; The United States Preventive Services Task Force; Cancer Intervention Surveillance Modeling Network; Fecal immunological technique; Modeling; Interval

Core tip: Multi-target stool DNA (mt-sDNA) testing was approved for average risk colorectal cancer (CRC) screening by the United States Food and Drug Administration (2014). The evidence supporting mt-sDNA for routine screening use is robust. The clinical efficacy of mt-sDNA every three years, measured by life-years gained, and CRC deaths averted, is similar to that of other screening strategies more specifically recommended by the United States Preventive Services Task Force. In an irregularly screened population, however, tests with the highest point sensitivity and reasonable specificity like mt-sDNA are more likely to reduce CRC related morbidity and mortality than less sensitive tests that depend on annual adherence to achieve high programmatic sensitivity.

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INTRODUCTION

In its October 5, 2015 draft recommendation statement for colorectal cancer (CRC) screening, the United States Preventive Services Task Force (USPSTF) includes multi-target stool DNA (mt-sDNA) as an "alternative" screening test that "may be useful in select clinical circumstances"[1]. However, the evidence supporting mt-sDNA for routine screening use is robust. The clinical efficacy of mt-sDNA as modeled for life-years gained (LYG), and CRC deaths averted is similar to other options more specifically recommended in the draft statement. This paper reviews the evidence supporting mt-sDNA clinical validity[2-4], analytical validity[5-7], and the USPSTF CRC screening modeling[8-10]. This body of evidence supports the use of mt-sDNA at three-year intervals (mt-sDNA3y) to provide significant clinical net benefits and fewer complications per LYG than annual fecal immunochemical testing (FIT), annual high sensitivity guaiac based fecal occult blood testing (hsFOBT), and screening colonoscopy every 10 years (Colo10y). In comparison to biennial or triennial FIT (FIT2y, FIT3y) or hsFOBT (hsFOBT2y, FSFOBT3y), both of which are adherence intervals more typically achieved in clinical practice[11,12], only mt-sDNA3y is at or within 98% of the screening test efficiency frontier[10] as measured by the ratio of LYG to colonoscopies generated. Additionally, mt-sDNA3y generates greater than 90% of the LYG by Colo10y in the simulation model of colorectal cancer (SimCRC)[10].

CRC is the second leading cause of cancer death in the United States. In 2015, an estimated 133000 people will be diagnosed with the disease and about 50000 will die from it[13]. When detected early, CRC can be treated with positive outcomes. The Centers for Disease Control and Prevention estimate that 42% of Americans are not currently up to date with colon cancer screening, including millions of Americans who have avoided screening completely, and that if everyone age 50 or older was regularly screened, at least 60% of CRC deaths could be avoided[14]. These statistics leave much room for improvement in routine CRC screening; effective, broad implementation of a non-invasive high sensitivity, low risk screening strategies like mt-sDNA could immediately help to address this serious public health issue.

THE mt-sDNA TEST

mt-sDNA uses a single random stool sample, collected by patients at home, without requiring any preparation, or change in medications or diet. The test identifies 10 biomarkers known to be associated with CRC and pre-cancerous lesion, including altered human DNA and hemoglobin. The test combines all biomarker results with the normalizing gene beta-actin in an algorithm that generates a composite, single, "Negative" or "Positive" patient result. Several detailed reviews describing the biology that underlies this screening approach and the design features of the test have been published[5-7]. The test was systematically designed to lower patient burdens with respect to ease of sample collection and specimen handling with a custom-designed collection kit. High CRC, high-grade dysplasia, and large adenoma point sensitivity and the prolonged pre-malignant phase of colorectal carcinogenesis allow for longer screening intervals between screening tests with mt-sDNA, which significantly lowers the burdens of annual testing. The American Cancer Society currently recommends mt-sDNA at three-year intervals[15]. Burdens on patients, providers and health care systems are further reduced through an included United States 24 h, 7 d-a-week telephonic patient navigation system. This system assists patients throughout the testing process to maximize the number of successful screening events and reports results to directly to ordering medical providers.

The mt-sDNA analytic process and biomarker selection were used to create a test with greater sensitivity for CRC and significant premalignant lesions than fecal hemoglobin as a single marker. All tests based on fecal hemoglobin alone are biologically limited in their ability to detect colorectal neoplasia, especially precancerous lesions and early stage CRC, which may bleed intermittently or not at all. The random sampling and small sample size used in FIT and hsFOBT tests contribute to sampling error and further limits detectability. In contrast, mt-sDNA detects DNA alterations, both mutations and aberrant methylation, in DNA released from cells that are constantly shed from premalignant lesions and early and late stage cancers, enhancing their detectability by mt-sDNA. Stool sampling error with mt-sDNA is significantly diminished through the

use of an aliquot of the entire stool sample homogenate and the laboratory's automated, uniform processing protocol[2].

mt-sDNA is primarily being used in the United States and has been approved by the United States Food and Drug Administration for average risk CRC screening[16] and is reimbursed by the United States Centers for Medicare and Medicaid Services once every three years[17]. It was awarded a unique Clinical Procedural Terminology code of 81528 by the American Medical Association. Cologuard® (multi-target sDNA) has been CE marked for use in Europe, though there is limited availability to date through laboratories in England and Dubai. Additional studies to determine local efficacy in a number of Asian countries are under discussion and studies are ongoing in Italy, the United Kingdom and the Netherlands. The test cost is USD649 (USD509 for Medicare), which includes a United States patient navigation/compliance system supporting over 70 languages.

In the United States, mt-sDNA is used in both opportunistic and local invitational settings. The test, requiring no change in diet, medication or any preparation and a specimen collection at home, is appropriate for population-based use. However, the size of the specimen container, allowable 72 h transit time back to the laboratory, and cost may mitigate use in that manner, especially in low resource countries. Countries with limited colonoscopy capacity for evaluating positive tests may be challenged by somewhat lower specificity (90%) of mt-sDNA compared to FIT, measured in patients requiring no biopsies when examined with colonoscopy, in any given year. However, overall, mt-sDNA3y results in fewer negative colonoscopic follow-up examinations for a positive screening test than result from the compounded programmatic specificity failure of annual FIT or hsFOBT at 95% specificity[8].

mt-sDNA PERFORMANCE IN SCREENING POPULATIONS

Three studies in screening populations show consistent results. Studies demonstrate significant greater single test application sensitivity of mt-sDNA over FIT for advanced colorectal neoplasia detection, though at lower specificity.

DeeP-C study

Results from this pivotal, prospective 90-site, 10000 patient cross-sectional clinical study were published in the New England Journal of Medicine in April 2014[2]. The DeeP-C study compared mt-sDNA and FIT, using colonoscopy as the reference standard on all cases. The study demonstrated that mt-sDNA was significantly more sensitive than FIT (Table 1) for detecting CRC, especially early stage CRC and advanced and non-advanced adenomas. Specificity in the target screening age of 50-74 years was 92.3% compared to 97.0% for FIT in patients where no biopsy was required during colonoscopy. Matching FIT specificity to that of the mt-sDNA test only increases FIT sensitivity 2 percentage points. Thus the lower specificity of mt-sDNA did not account for the increased sensitivity of the mt-sDNA assay over FIT.

Alaska study: This prospective study of 661 Alaska native people compared mt-sDNA to FIT with colonoscopy as the reference, the same design as DeeP-C[3]. In the overall study population (n = 661), which included both higher risk and average risk individuals for routine screening (screen subgroup) mt-sDNA detected 49% of advanced colorectal neoplasms (colorectal cancer plus advanced adenoma) *vs* 28% for FIT ($P < 0.001$), including the identification of 100% (10/10) colorectal cancers *vs* 80% (8/10) for FIT. In the screen sub-group (n = 464), mt-sDNA detected 50% advanced colorectal neoplasia *vs* 31% for FIT ($P = 0.01$), including the detection of 100% (4/4) of colorectal cancers *vs* 75% (3 of 4) for FIT. In subjects with no adenomas detected on colonoscopy, specificity was 93% for mt-sDNA *vs* 96% for FIT ($P = 0.034$). mt-sDNA may provide an attractive approach to provide highs sensitivity screening for populations where routine travel for colonoscopy is challenging or where colonoscopy capacity itself is limited. Similarly, individuals participating only irregularly in screening care may accrue a greater benefit using a higher sensitivity tests when provided with an opportunity for screening.

Netherlands study: mt-sDNA was compared to FIT (OC Sensor) in prospectively collected frozen archived samples (n = 1047)[4] from an invitational screening cohort (COCOS) collected in the Netherlands[18]. The study compared the performance of mt-sDNA and FIT to colonoscopy on all subjects for the detection of advanced colorectal neoplasia. mt-sDNA detected 49% (50/102) and FIT 25% (26/102) of cases of advanced colorectal neoplasia ($P < 0.001$) at specificities of 89% and 96% respectively. The findings are consistent with those of the DeeP-C and Alaska studies.

PATIENT PREFERENCES AND TEST PERFORMANCE IN DIFERENT POPULATIONS AFFECT SCREENING PROGRAM EFFICACY

The effectiveness of a test is a function of its performance, its availability, and patient adherence. The USPSTF noted in their draft statement that "clinicians should consider engaging patients in informed decision-making about the screening strategy that would most likely result in completion, with high adherence over time, taking into consideration both the patient's preferences and local availability"[1]. Data has begun to accrue from studies that the mt-sDNA performance and process may appeal to previously unscreened patients

Table 1 Findings from the DeeP-C cross-sectional study[5], comparing multitarget stool DNA with fecal immunological test using colonoscopy as the reference standard on all cases (*n* = 9989 subjects)

	Colonoscopy findings *n* detected	mt-sDNA % detected	FIT % detected
Sensitivity			
Colorectal cancer (stages Ⅰ-Ⅳ)	65	92.3%	73.8%
Early stage colorectal cancer (stage Ⅰ and Ⅱ)	50	94.0%	70.0%
AA	757	42.4%	23.8%
High grade dysplasia	39	69.2%	46.2%
Sessile serrated adenoma/polyp ⩾ 1.0 cm	99	42.4%	5.1%
Specificity			
Specificity (only CRC and AA excluded)	9167	86.6%	94.9%
Specificity, no adenomas, no biopsy done	4457	89.8%	96.4%
Age-adjusted (50-74 yr)[31]	4032	92.3%	97.0%

FIT: Fecal immunological test; AA: Advanced adenoma.

and lead to increased screening rates. A study by Berger *et al*[19] surveyed a random sample of almost 3000 average-risk patients (99% participation rate) who had been prescribed mt-sDNA (Cologuard) by their physician and found that 42% of the patients, aged 50-74 years had not been previously screened.

A second study by Cole *et al*[20] of 675 average-risk patients, aged 50-75 years who had never been screened for CRC showed that when patients were informed regarding screening alternatives they preferred the noninvasive, mt-sDNA option by more than 50% over colonoscopy or FIT. The study went on to note that educating patients about the noninvasive mt-sDNA option and involving the patient in the shared decision-making process about test choice can increase the likelihood that noncompliant patients will get screened.

A third study by Abola *et al*[21] of 423 individuals (617 invited, 69% participation rate) found that 75% considered mt-sDNA more suitable for screening than colonoscopy with no significant difference between Caucasian and African American respondents. The authors concluded that "intervention to increase the uptake of sDNA (mt-sDNA) testing may reduce racial disparities in CRC".

The availability of mt-sDNA allows patients who would be screened but who will not use colonoscopy for personal or cultural reasons or who reside in rural areas where there is less access to colonoscopy, to have a high sensitivity noninvasive test option. This is especially relevant for those who will not adhere to an annual screening regime.

Addressing disparities resulting from test access and patient preference are important. Studies show that multiple screening choices increase the overall level of screening and that patients will gravitate to their preferred option. Inadomi *et al*[11] showed that offering colonoscopy alone was less effective (38% screened) than offering a choice of colonoscopy or gFOBT screening (69% screened) for obtaining a successful screening event within 12 mo of a physician's recommendation for screening. A large prospective randomized screening study comparing FIT to colonoscopy, conducted in Spain, by Quintero *et al*[22], allowed post-randomization cross-over. Approximately 23% (1706/7355) of patients randomized to colonoscopy crossed over to FIT, with 1.2% (117/9353) of patients randomized to FIT crossing over to colonoscopy. This demonstrates a preference for non-invasive testing in a significant number of subjects. If this occurred in a clinical situation outside of a clinical trial, 23% of patients would be selecting a screening approach with much less sensitivity and a more burdensome screening requirement. These issues could be significantly mitigated by using high sensitivity mt-sDNA as a routine non-invasive choice. Additional studies have shown that test preferences for non-invasive screening over invasive screening are most attractive to certain populations related to cultural preferences, a population area where high sensitivity non-invasive screening may also improve screening efficacy.

Finally, for patients who do choose non-invasive testing, sensitivity for CRC and its precursors in the proximal colon are key to screening efficacy. African American patients and elderly patients in general have an increased prevalence of proximal colorectal neoplasia[23-25]. In contrast to FIT which is more sensitive for lesions in the distal colon, mt-sDNA has equivalent sensitivity for CRC in the proximal and distal colon. Importantly, and key for decreasing proximal CRC incidence when using noninvasive screening, mt-sDNA has significant sensitivity for sessile serrated adenoma/polyps, the pre-cursor lesion for approximately 25% of CRC and a common cause of missed or interval cancers arising in the proximal colon. In the DeeP-C study, mt-sDNA identified 42.4% of patients with sessile serrated adenomas ≥ 1 cm in diameter whereas FIT detected only 5.1% ($P < 0.001$) as these lesions are non-hemorrhagic whereas they exfoliate aberrantly methylated DNA in the stool[2].

MODELING mt-sDNA PERFORMANCE AS AN APPROACH FOR SETTING AN INITIAL INTER-TEST INTERVAL

Establishing an initial inter-test interval using vetted, well designed and calibrated CRC screening models is recommended for new tests[8]. Modeled intervals can be

tempered over time to accommodate accumulating clinical experience, patient preferences and medical delivery system impacts. Comparative prospective randomized longitudinal studies with mortality endpoints are large, complex, lengthy, and expensive. Currently, only screening with low sensitivity guaiac FOBT and flexible sigmoidoscopy are supported by prospective randomized control trials (RCT's) with mortality endpoints. The use of models allows for virtual prospective studies to be done on large cohorts. These provide comparative performance of multiple tests simultaneously. Such modeling and its limitations are described below.

The predictive power of modeling has limitations related to the degree that the biology of colorectal cancer, clinical practice related factors, test uptake, and performance assumptions accurately reflect the clinical screening "ecosystem". One concern is establishing a comparative baseline by only using 100% uptake and adherence for each screening test. The USPSTF technical report[8] assumes 100% compliance and adherence for each strategy, despite strong evidence that initial uptake of FIT/FOBT in the United States is low (10.4%)[26,27] and adherence for FIT and FOBT declines significantly over time[28]. A recent review of a large cohort of patients continuously insured for ten years showed that of patients who were screened according to guidelines, only 0.3% (268/97518) were current with screening as a result of completing ten consecutive annual FIT/FOBT tests[29]. Patients who were non-adherent with colonoscopy and non-adherent to annual test use completed an average of 2.6 FIT/FOBT during the 10-year study period. Forty-six percent completed only a single FOBT/FIT test during the 10-year study period. Ninety-nine point six percent (97801/97518) of patients who were current with screening had received a colonoscopy during the 10-year study period. In a three-year follow-up[28] of the Inadomi study[11] reported by Liang *et al*[28], the annual adherence for patients in the group assigned to FOBT screening fell from 67% the first year to 27% at year two, and to 14% by year three. In the group that chose FOBT over colonoscopy, adherence dropped from 38% in the first year to 19% at year two and to 12% at year three. In highly resourced integrated health system with patient navigation infrastructure, improved programmatic adherence with annual FIT has been shown, though initial uptake remains < 50%[30]. Despite evidence of poor year-over-year adherence, the USPSTF continues to recommend routine screening with annual FIT, hsFOBT, or annual FIT with flexible sigmoidoscopy every 10 years, based on modeling 100% adherence while acknowledging that "In practice, such high adherence is not observed either for initial or repeat screening[8]" and considers high sensitivity mt-sDNA an "alternative test" only for use in selected patients.

This USPSTF draft CRC screening recommendation[1] is informed by the results of three independently-developed microsimulation models of CRC that are funded by the National Cancer Institute's Cancer Intervention and Surveillance Modeling Network (CISNET) - SimCRC, microsimulation screening analysis (MISCAN) for CRC, and colorectal cancer simulated population model for incidence and natural history. The performance of the various CRC screening tests were evaluated using these models to predict LYG, decreases in CRC incidence, CRC related mortality, number of screening tests required, and complications arising from screening[8].

For comparative purposes, CISNET assumed 100% perfect adherence to all screening and surveillance procedures and performed no sensitivity analysis around adherence that would more accurately reflect actual test use. Further, their analysis grouped FIT, gFOBT, and mt-sDNA together because they are "exclusively stool-based screening modalities with comparable burden"[8]. However, mt-sDNA has higher single-event CRC, and advanced adenoma sensitivity, including sensitivity for sessile serrated adenomas. It has significantly lower patient burdens given the need for far fewer test events with the recommended three-year screening schedule, a specifically designed patient collection process to minimize sample handling, and an embedded patient navigation support system. Based on the lower patient burden and the great biological differences in the test approach, mt-sDNA could have been considered in its own category for interval effect analysis, similar to all other non-stool based strategies. At the least, mt-sDNA3y could have been grouped with FIT2y and FIT 3y and hsFOBT2y and hsFOBT3y as a more clinically representative grouping for evaluation. This is an important consideration as, under CISNET modeling rules, only one strategy per "group" could ultimately be "recommended" for routine screening[8].

This grouping and arbitrary rule led to the finding that "annual mt-sDNA" was less efficient with respect to the number of colonoscopies generated per LYG than annual FIT and hsFOBT and precluded a consideration of the multi-year interval (3 years) for which the test is already recommended by others[9,15]. According to the draft recommendation statement, the CISNET modeling of mt-sDNA at a one-year interval (mt-sDNA1y) would "potentially yield approximately the same number of life-years gained as the recommended strategies previously listed" but when "compared with other stool-based screening tests and screening with colonoscopy every 10 years, FIT-DNA (mt-sDNA) requires a larger number of lifetime colonoscopies (a proxy for the harms of screening) per LYG"[1]. When calculating lifetime colonoscopies using the intervals for each screening test as recommended by the American Cancer Society (Table 2)[15], the data shows that mt-sDNA3y has the fewest lifetime colonoscopies (COL) and an equivalent number of colonoscopies per LYG compared to FIT and hsFOBT at one-year intervals (FIT1y and hsFOBT1y) (Table 3)[8]. Colonoscopy itself generates approximately twice as many colonoscopies per LYG as any of the non-invasive strategies.

Overall, mt-sDNA3y is associated with less burdens and harms than FIT1y and gFOBT1y. In clinical practice the comparative benefits of mt-sDNA may be even greater given the lack of adherence to annual FIT or

Table 2 American Cancer Society recommended colorectal cancer screening test frequency intervals for average risk individuals

Test	Frequency (yr)
Colonoscopy	10
CT colonography	5
Flexible sigmoidoscopy	5
Multi-target stool DNA test (Cologuard, mt-sDNA)	3
High sensitivity guaiac-based fecal occult blood test	1
Fecal immunochemical test	1

CT: Computed tomography.

hsFOBT screening[11,28-30]. A comparison reflecting actual clinical practice experience would have included a comparison of mt-sDNA3y with FIT/FOBT at 2y and 3y, which is detailed below.

A sensitivity analysis exploring non-annual adherence demonstrated more clinically relevant benefits and harms for stool-based strategies. The CISNET modeling data on FIT and hsFOBT at two-year intervals[8] (FIT2y and hsFOBT2y) and mt-sDNA3y (Table 4), show mt-sDNA3y to be the only strategy generating greater than 90% LYG by screening colonoscopy 10y (% of COL 10y LYG) (SimCRC) in any of the models. While the colonoscopies per LYG are similar for hsFOBT and somewhat lower for FIT2y, overall LYG, CRC incidence, and related deaths are notably lower and more lives will be saved with mt-sDNA3y than with either FIT2y or gFOBT2y[8].

The CISNET modeling data on FIT and gFOBT at three-year intervals[8] (FIT3y, hsFOBT3y) reflects a second scenario supported by clinical experience[8-10]. Compared to FIT3y and hsFOBT3y, mt-sDNA benefits are notably better with 19-22 CRC deaths averted, 43%-68% CRC incidence reduction, and 68%-78% mortality reduction across the three models (Table 5)[8]. At three-year intervals, FIT and hsFOBT generate only 68%-77% of the life years gained by Colo10y *vs* 84%-91% for mtsDNA3[8,10].

BALANCING BENEFITS AND HARMS

The specific harms associated with the non-invasive testing process are held to be minimal. Paradoxically, the USPSTF[1] uses colonoscopies as a proxy for harms for the non-colonoscopy screening tests, but not for colonoscopy based screening itself. If colonoscopy related harm is a greater concern than screening benefit, especially where differences in the balance of harms and benefits is very small among non-invasive tests, mt-sDNA3y appears favorable when compared to Colo10y. mt-sDNA3y generates far fewer colonoscopies per 1000 people screened (1701-1827) across the three CISNET models[8] than Colo10y (4007-4101) or annual hsFOBT (2230-2287) and similar numbers to annual FIT (1739-1899) (Table 3)[8].

There are no direct harms or complications from mt-sDNA beyond those associated with a follow-up colonoscopy for a positive mt-sDNA screening test. No additional investigation is indicated for a positive mt-sDNA test outside a careful structural examination of the colon in a well prepared patient, generally by optical colonoscopy. The aggregate contribution of other cancers of the aerodigestive tract and inflammatory diseases to the mt-sDNA false positive rate is two cases per 10000 screened patients, precluding the need for additional studies in an otherwise asymptomatic patient on the basis of a positive mt-sDNA test alone[31]. Like all tests, mt-sDNA may be associated with false positive results and false negative results, wherein advanced colorectal neoplasia is not identified on a single screening event. Colonoscopy, however, may be associated, though rarely, with significant adverse events[31].

The USPSTF technical report calculated complications for all model outputs. These complications are based on the serious adverse event rates summarized in the USPSTF evidence synthesis[25] and are dependent on patient age and type of lesion removed. Table 3 shows the complications per 1000 patients screened, the LYG, the CRC deaths averted and screening related complications across the three models. mt-sDNA3y has the lowest rate of complications per LYG (0.036-0.044) *vs* annual FIT (0.038-0.045), annual hsFOBT (0.042-0.047) or colo10y (0.051-0.060). With respect to complications per death averted, mtsDNA3y (0.41-0.50) outperforms colonoscopy (0.58-0.68) and in two of three models, annual FIT (0.43-0.50) and annual hsFOBT (0.48-0.55)[8].

Finally, the total number of screening tests required itself is an indicator burden. Fewer stool tests and clinical encounters are required for mt-sDNA3y than with FIT1-3y or hsFOBT1-3y (Tables 3-5). mt-sDNA3y provides significantly fewer burdens on patients, physicians, and healthcare systems than other fecal tests. Notably, this factor was not accounted for as a "burden" in the USPSTF analysis[8].

mt-sDNA CLINICAL UTILITY CAN BE INFERRED FROM PREVIOUS RCT'S OF FOBT

The clinical utility of mt-sDNA3y with respect to reducing both CRC related mortality and CRC incidence can be inferred from previous RCT's of annual and biennial screening with the less sensitive FOBT test. No CRC screening test recommended by the USPSTF has been shown empirically to decrease CRC related mortality[25]. Only low sensitivity guaiac based FOBT (gFOBT, *e.g.*, Hemoccult Ⅱ), used annually or biennially, and flexible sigmoidoscopy alone have been shown to decrease CRC mortality in well-designed RCTs, but these are no longer widely used in the United States for screening, nor recommended by the USPSTF[1,8,25]. However, the USPSTF infers decreases in CRC related mortality and the CRC incidence for both FIT and hsFOBT from the mortality benefit demonstrated for Hemoccult Ⅱ gFOBT in these RCT's[8]. These benefits can also be applied to mt-sDNA similarly through the same logical inference, as given mt-sDNA's superior sensitivity over FIT (Table 1) and by

Table 3 Burdens, harms, benefits, and efficiencies for 100% perfect adherence for colorectal cancer screening tests at current recommended intervals, ages 50-75, per 1000 people screened

CISNET model	Test	Burdens and harms			Benefits				% of COL 10y LYG	COL per LYG	Complications per LYG	Complications per DA
		Stool tests	Total COL	Complications	LYG	CRC DA	CRC incidence reduction	CRC mortality reduction				
SimCRC	COL 10y	0	4007	14	275	24	81%	87%	100%	15	0.051	0.58
	FIT1y	15778	1739	10	260	23	67%	81%	95%	7	0.038	0.43
	hsFOBT1y	12914	2230	11	261	23	69%	82%	95%	9	0.042	0.48
	mt-sDNA3y	5990	1701	9	250	22	63%	78%	91%	7	0.036	0.41
MISCAN	COL 10y	0	4101	15	248	22	62%	79%	100%	17	0.060	0.68
	FIT1y	15843	1757	10	231	20	47%	72%	93%	8	0.043	0.50
	hsFOBT1y	12927	2287	11	232	20	49%	73%	94%	10	0.047	0.55
	mt-sDNA3y	5779	1714	9	215	19	43%	68%	87%	8	0.042	0.47
CRC-SPIN	COL 10y	0	4049	15	270	24	88%	90%	100%	15	0.056	0.62
	FIT1y	15444	1899	11	244	22	72%	81%	90%	8	0.045	0.50
	hsFOBT1y	13026	2253	11	247	22	75%	82%	92%	9	0.045	0.50
	mt-sDNA3y	5927	1827	10	226	20	68%	76%	84%	8	0.044	0.50

CISNET: National Cancer Institute's Cancer Intervention and Surveillance Modeling Network; SimCRC: Simulation model of colorectal cancer; MISCAN: Microsimulation screening analysis; CRC-SPIN: Colorectal cancer simulated population model for incidence and natural history; COL: Colonoscopies; LYG: Life-years gained; DA: Deaths averted; COL 10y: Colonoscopy at a 10-year interval.

Table 4 Burdens, harms, benefits, and efficiencies at 2-year adherence rates for fecal immunological technique/fecal occult blood testing compared to recommended intervals for colonoscopy and mt-sDNA, ages 50-75, per 1000 people screened

CISNET model	Test	Burdens and harms			Benefits				% of COL 10y LYG	COL per LYG	Complications per LYG	Complications per DA
		Stool tests	Total COL	Complications	LYG	CRC DA	CRC incidence reduction	CRC mortality reduction				
SimCRC	COL 10y	0	4007	14	275	24	81%	87%	100%	15	0.051	0.58
	FIT2y	9326	1215	7	234	20	53%	72%	85%	5	0.030	0.35
	hsFOBT2y	8388	1597	9	235	21	56%	73%	86%	7	0.038	0.43
	mt-sDNA3y	5990	1701	9	250	22	63%	78%	91%	7	0.036	0.41
MISCAN	COL 10y	0	4101	15	248	22	62%	79%	100%	17	0.060	0.68
	FIT2y	9342	1243	8	200	17	35%	62%	81%	6	0.040	0.47
	hsFOBT2y	8408	1636	9	200	18	37%	63%	81%	8	0.045	0.50
	mt-sDNA3y	5779	1714	9	215	19	43%	68%	87%	8	0.042	0.47
CRC-SPIN	COL 10y	0	4049	15	270	24	88%	90%	100%	15	0.056	0.62
	FIT2y	9241	1346	9	207	18	58%	68%	77%	6	0.043	0.50
	hsFOBT2y	8448	1626	9	212	19	62%	70%	78%	8	0.042	0.47
	mt-sDNA3y	5927	1827	10	226	20	68%	76%	84%	8	0.044	0.50

CISNET: National Cancer Institute's Cancer Intervention and Surveillance Modeling Network; CRC: Colorectal cancer; SimCRC: Simulation model of CRC; MISCAN: Microsimulation screening analysis; CRC-SPIN: Colorectal cancer simulated population model for incidence and natural history; COL: Colonoscopies; LYG: Life-years gained; DA: Deaths averted; COL 10y: Colonoscopy at a 10-year interval; FIT: Fecal immunological test.

inference, superiority to low sensitivity gFOBT[2]. CISNET modeling supports the proposition that mt-sDNA3y is more efficient[10] and efficacious than hsFOBT2y and therefore similar clinical utility can be ascribed to mt-sDNA3y as the four RCTs of low sensitivity gFOBT2y provide for the clinical utility of hsFOBT2y[8]. Using comparable clinical efficiency[10] to allow clinical utility to be inferred from the RCTs of biennial FOBT obviates concern around small differences in specificity between the tests. In practical terms, even if patient uptake of mtsDNA is the same as that of FIT or hsFOBT, patients are more likely to see a greater net benefit from higher sensitivity mt-sDNA screening than from intermittent FIT/FOBT use, especially given the low rate of serious colonoscopy related complications.

CONCLUSION

CRC is the second leading cause of cancer mortality in the United States with nearly 50000 deaths per year. mt-sDNA provides a colorectal cancer screening test with high sensitivity for the detection of CRC and the most significant pre-malignant lesions in a non-invasive format. It is approved as safe and effective for routine screening of asymptomatic individuals by the United States FDA, CE marked in Europe, and vetted and approved for coverage by the United States Centers of Medicare and Medicaid Services at three year intervals. mt-sDNA at three-year intervals is included in the American Cancer Society guidelines. Clinical experience demonstrates that patients formerly non-compliant with screening, ages 50-74, comprise a significant proportion (42%) of mt-sDNA users, which is consistent with patient screening preference studies.

Multiple studies support the superior point sensitivity of mt-sDNA over FIT, an important attribute of a screening test with limited harms that appeals to patients hesitant to pursue screening by other methods. The data

Table 5 Burdens, harms, benefits, and efficiencies for fecal immunological technique and fecal occult blood testing at 3-year adherence rates compared to recommended intervals for colonoscopy and mt-sDNA, ages 50-75, per 1000 people screened

		Burdens and harms			Benefits							
CISNET model	Test	Stool tests	Total COL	Complications	LYG	CRC DA	CRC incidence reduction	CRC mortality reduction	% of COL 10y LYG	COL per LYG	Complications per LYG	Complications per DA
SimCRC	COL 10y	0	4007	14	275	24	81%	87%	100%	15	0.051	0.58
	FIT3y	6887	971	6	212	18	45%	65%	77%	5	0.028	0.33
	hsFOBT3y	6456	1286	7	212	18	47%	66%	77%	6	0.033	0.39
	mt-sDNA3y	5990	1701	9	250	22	63%	78%	91%	7	0.036	0.41
MISCAN	COL 10y	0	4101	15	248	22	62%	79%	100%	17	0.060	0.68
	FIT3y	6795	995	7	176	15	28%	55%	71%	6	0.040	0.47
	hsFOBT3y	6302	1296	8	175	15	30%	55%	71%	7	0.046	0.53
	mt-sDNA3y	5779	1714	9	215	19	43%	68%	87%	8	0.042	0.49
CRC-SPIN	COL 10y	0	4049	15	270	24	88%	90%	100%	15	0.056	0.62
	FIT3y	6857	1081	7	178	16	49%	59%	66%	6	0.039	0.44
	hsFOBT3y	6498	1317	8	183	16	53%	61%	68%	7	0.044	0.50
	mt-sDNA3y	5927	1827	10	226	20	68%	76%	84%	8	0.044	0.50

CISNET: National Cancer Institute's Cancer Intervention and Surveillance Modeling Network; COL: Colonoscopies; COL 10y: Colonoscopy at a 10-year interval; LYG: Life-years gained; CRC: Colorectal cancer; MISCAN: Microsimulation screening analysis; CRC-SPIN: Colorectal cancer simulated population model for incidence and natural history; SimCRC: Simulation model of colorectal cancer; DA: Deaths averted; FIT: Fecal immunological test.

provided by the USPSTF technical report[8] from three separate models supports the efficacy of mt-sDNA3y and demonstrates across 1000 screened individuals, age 50-74, that it yields a median of 226 life-years gained (range 215-250), averts 20 CRC deaths (range 19-22), reduces CRC mortality by 76% (range 68%-78%, and produces the most benefit (LYG) per complication (harm). The three CISNET models demonstrate that in terms of the number of colonoscopies per LYG. mt-sDNA3y (7-8) is equivalent to annual FIT (7-8) and lower than hsFOBT (9-10)[8].

The USPSTF draft recommendation states "Screening for CRC is a substantially underused preventive health strategy in the United States… Accordingly, the best screening test is the one that gets done" and that maximizing the total proportion of the eligible population that receives screening will "result in the greatest reduction in deaths due to CRC"[1]. As such, the clinical and modeling evidence and societal need for improved noninvasive screening strategies support a USPSTF recommendation for mt-sDNA for routine screening at three-year intervals, a conclusion consistent with the recommendations of others.

Failure to include a clear recommendation of mt-sDNA3y in the final USPSTF guideline may limit access to mt-sDNA for Americans not covered by Medicare. By only recommending the same tests as were recommended in 2008, the USPSTF draft recommendation limits significant progress in improving United States screening rates[13,14] by affirming the current approaches only. In order to increase the screening rate, we must offer more efficacious choices. mt-sDNA3y for routine screening provides an opportunity to expand the pool of screened patients and to increase the quality of screening among those choosing non-invasive approaches.

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MINIREVIEWS

Urinary metabolites as noninvasive biomarkers of gastrointestinal diseases: A clinical review

Irene Sarosiek, Rudolf Schicho, Pedro Blandon, Mohammad Bashashati

Irene Sarosiek, Mohammad Bashashati, Division of Gastroenterology, Department of Internal Medicine, Texas Tech University Health Sciences Center, El Paso, TX 79905, United States

Rudolf Schicho, Institute of Experimental and Clinical Pharmacology, Medical University of Graz, 8010 Graz, Austria

Pedro Blandon, Division of Nephrology, Department of Internal Medicine, Texas Tech University Health Sciences Center, El Paso, TX 79905, United States

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Correspondence to: Mohammad Bashashati, MD, Research Scientist, Division of Gastroenterology, Department of Internal Medicine, Texas Tech University Health Sciences Center, 4800 Alberta Ave, El Paso, TX 79905,
United States. bashashati.md@gmail.com
Telephone: +1-915-2155148
Fax: +1-915-5456210

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Abstract

The diagnosis of gastrointestinal (GI) disorders is usually based on invasive techniques such as endoscopy. A key important factor in GI cancer is early diagnosis which warrants development of non- or less-invasive diagnostic techniques. In addition, monitoring and surveillance are other important parts in the management of GI diseases. Metabolomics studies with nuclear magnetic resonance and mass spectrometry can measure the concentration of more than 3000 chemical compounds in the urine providing possible chemical signature in different diseases and during health. In this review, we discuss the urinary metabolomics signature of different GI diseases including GI cancer and elaborate on how these biomarkers could be used for the classification, early diagnosis and the monitoring of the patients. Moreover, we discuss future directions of this still evolving field of research.

Key words: Metabolomics; Gastrointestinal diseases; Cancer; Inflammatory bowel disease; Metabolome; Urine

Core tip: Scientists are always searching for new disease biomarkers. An acceptable biomarker could help us in early diagnosis and classification of the diseases as well as the prediction of disease outcome. The diagnosis of gastrointestinal (GI) diseases is usually based on techniques such as upper or lower GI endoscopy, while highly sensitive and specific non-invasive diagnostic or screening tools are usually lacking. In this review, we have discussed the potentials of urinary metabolomics study as a future tool for the screening, diagnosis, classification and surveillance of GI diseases including inflammatory bowel disease and cancer.

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INTRODUCTION

The rapid growth of high-quantity technologies and computational contexts allows the analysis of organic systems in distinctive details. New technologies such as DNA sequencing and mass spectrometry have permitted observing thousands of molecules concurrently instead of a few components that have been analyzed in old-fashioned research[1].

By considering epigenetic ruling and posttranslational alterations, metabolites serve as direct signatures of biochemical activity in biological systems. Moreover, beyond genes and proteins, they are usually in direct association with disease phenotypes[2]. Metabolomics or metabolic profiling is based on comprehensive and rapid analysis of thousands of metabolites simultaneously in biological samples including plasma and urine and is a feasible strategy for biomarker discovery[3].

The routine urine analysis is often used for the diagnosis of diseases in the urinary tract. However, more than 3000 metabolites are detectable in the urine and their levels may be used as the signature of systemic diseases[4]. These signatures are affected by energy and nutrient intake, body and cellular metabolisms and the environmental factors such as microbiota which have close cross-talk with the gastrointestinal (GI) system. Therefore, any disease in the GI tract may change the metabolic profile of the body that can be reflected in the bodily fluids including blood and urine.

This review provides an insight to the urinary metabolic profile of the GI diseases and its potential application in the clinical diagnosis and predicting their clinical as well as treatment outcome.

TECHNIQUES AND EVALUATION OF URINARY METABOLOMES

Assessment of some GI diseases requires the use of endoscopic methods which are not without risks. Determination of disease biomarkers in easily obtainable biofluids like urine, therefore, would be a valuable adjunct or even an alternative to conventional methods. Many serological markers for inflammatory bowel diseases (IBD) already exist, however, they are less helpful in determining disease subtypes (*i.e.*, Crohn's disease and ulcerative colitis) or forms of indeterminate colitis[5]. Biomarkers or biomarker profiles that can predict and discriminate these subtypes with high probability are therefore desirable. Various studies pursuing this goal have been performed in the past couple of years and have increased the list of metabolites found in higher or lower concentrations in body fluids, including urine, during IBD[6]. These metabolites have been measured in IBD patients by highly sensitive techniques, for instance, by 1H nuclear magnetic resonance (NMR) spectroscopy[7-9], ion cyclotron resonance-Fourier transform mass spectrometry[10] and by ultra-performance liquid chromatography coupled with electrospray ionization quadruple time-of-flight mass spectrometry (MS) in rodents with experimental colitis[11]. While the latter techniques are characterized as extremely sensitive, 1H NMR spectroscopy is maybe less sensitive but known to produce highly reproducible results. A recent study compared different techniques for the detection of urine metabolites in humans and concluded that the NMR technique is the best method for identifying and quantifying urinary compounds[4].

For the discrimination of IBD subtypes and the determination of severity and progress of GI diseases, many metabolites need to be identified. To organize and correctly interpret the large number of data, statistical methods, like multivariate analysis (*e.g.*, principal component analysis and orthogonal partial least squares projections) are applied. In the case of NMR, a method called "targeted or quantitative metabolic profiling" has been used to detect new possible sets of biomarkers[12]. Here, the spectra of already characterized metabolites are stored in a database, and spectra measured in a new biofluid sample are compared with those from the database and thus identified and quantified. The determined metabolites not only may have importance as potential biomarkers but they can, at the same time, provide a link to the pathophysiology of the disease. In this respect, knowledge on the role of the determined molecule within the metabolic pathway is important. A urine metabolome database that allows researchers access to the types, structures and concentrations of urinary metabolites in different diseases has been therefore introduced by the Metabolomics Innovation Centre (http://www.metabolomicscentre.ca/; a platform hosted by the University of Alberta, Canada)[4].

Taken together, urinary metabolites can be evaluated in GI diseases by different experimental methods of high or low sensitivity. Irrespective of the method used, detection of a unique metabolic fingerprint either for diagnosis, treatment, or detection of disease mechanisms is the primary goal.

URINARY METABOLOMICS IN IBD

IBD affecting over 1 million individuals in the United States and 2.5 million in Europe is a common chronic gastrointestinal disease with substantial costs for healthcare. Moreover, it is estimated that the absolute number of IBD patients in newly industrialized countries may approximate that in the Western world until 2025[13]. Despite this increase in the burden of IBD, gold standard tests for its diagnosis, monitoring and management are

usually invasive and sometimes inconclusive. Therefore, biomarkers including noninvasive methods such as urinary metabolomics studies might be useful for the management of patients with IBD[14,15].

Urinary metabolomics has been studied in different mouse models of IBD. Interleukin-10 (*IL-10*) gene-deficient mice which are genetically susceptible to inflammation and colitis have shown different urinary metabolomics compared to non-inflamed animals. For instance, Murdoch *et al*[16] showed that several urinary metabolites such as trimethylamine (TMA) and fucose are changed dramatically in the *IL-10* gene-deficient mice after 8 wk of age which is the timeline for development of severe histological injury and colitis. These alterations in the metabolomics are majorly mediated by commensal microflora which play a key role in the disease process.

In another study on *IL-10* gene deficient mice, Lin *et al*[17] showed an association between 15 metabolites including fucose, xanthurenic acid, and 5-aminovaleric acid with intestinal inflammation. Elevated urinary xanthurenic acid in gene deficient mice was linked to increased plasma levels of kynurenine[17]. In a further study, the same group validated these findings by showing that feeding *IL-10* gene-deficient and wild-type mice with Kiwifruit increases Kiwifruit-derived urinary metabolites more significantly in *IL-10* gene-deficient mice compared to wild-type mice without affecting urinary metabolites levels previously associated with inflammation[18].

In another study, Otter *et al*[19] showed association between the concentrations of xanthurenic acid, α-CEHC glucuronide, and an unidentified metabolite m/z 495(-)/497(+) with inflammation in *IL-10* gene deficient mice.

Overall, studies on *IL-10* gene deficient mice generally agree with changes in urinary xanthurenic acid, a product of tryptophan catabolism through the kynurenine pathway. Bacterial lipopolysaccharides and pro-inflammatory cytokines are the activators of this pathway and its metabolites act as the moderators of T-cell tolerance to intestinal microbiota. As colitis does not usually develop in germ-free *IL-10* gene deficient mice, the role of intestinal microbiota looks considerable in the induction of urinary metabolomics alterations during colitis[17,19-21].

Although, overall studies indicated that *IL-10* gene deficient mice have different urinary metabolomics profile compared to wild-type mice, Tso *et al*[22] showed that these differences are gender and age specific.

Schicho *et al*[23] expanded metabolomics study to an acquired model of chemical colitis induced by dextran sodium sulfate (DSS). After studying 69 urinary metabolites, they showed that urinary creatine, carnitine, and methylamines (including TMA and TMAO) were increased whereas antioxidant metabolites were decreased in DSS mice.

Another study on trinitrobenzene sulphonic acid-induced acute colitis in rats indicated that urinary tryptophan metabolites [4-(2-aminophenyl)-2,4-dioxobutanoic acid and 4,6-cihydroxyquinoline], gut microbial metabolites (phenyl-acetylglycine and p-cresol glucuronide), and the bile acid 12α-hydroxy-3-oxocholadienic acid which are associated with damage of the intestinal barrier function, microbiota homeostasis, immune modulation and the inflammatory response are altered during experimental colitis[11].

Moreover, in a naïve T cell adoptive transfer experimental model of colitis, Martin *et al*[24] showed decrease in Krebs cycle intermediates in urine (succinate, α-ketoglutarate) indicating reduction in the glutaminolytic pathway related to overall loss of energy homeostasis during colitis.

Besides studies on animal models of colitis, studies on IBD patients have confirmed the diagnostic potentials of urinary metabolomics. By studying 206 Caucasian subjects [86 Crohn's disease (CD) patients, 60 ulcerative colitis (UC) patients, and 60 healthy controls], Williams *et al*[9] showed that urinary metabolites, which were in correlation with intestinal microbiota, were different in IBD patients compared to controls. In brief, urinary hippurate differed significantly between the three groups with the lowest level in CD patients. Moreover, 4-cresol sulfate levels were lower and formate levels were higher in CD patients compared to UC patients or controls. This study could significantly differentiate CD from UC[9].

Another study compared the urinary metabolomic signature of patients with active UC, quiescent UC, and controls. In this study no significant difference in the urinary metabolomics profile of these 3 groups was observed[8]. On the other hand, based on a recent study, a significant partial least squares discriminant analysis model was obtained through measuring urinary metabolomics in patients with active IBD *vs* a group with IBD in remission. Based on this study, glycine was increased in urine and acetoacetate decreased in urine during active IBD. Moreover, in active IBD, urinary citrate, hippurate, trigonelline, taurine, succinate and 2-hydroxyisobutyrate were decreased compared to the controls. Despite mentioned observations, this study could not clearly differentiate CD and UC patients based on the analysis of urine samples. Interestingly, contrary to the serum samples, up-regulation of acetoacetate and down-regulation of citrate, hippurate, taurine, succinate, glycine, alanine and formate in the urine samples of patients with IBD in remission could distinguish them from healthy controls[25].

Another study showed that urinary metabolomics including tricarboxylic acid (TCA) cycle intermediates, amino acids, and gut microflora metabolites are different in patients with IBD compared to healthy controls. Comparison of CD and UC patients revealed different metabolomics fingerprints, but removal of patients with the surgical intervention revealed that CD could not be differentiated from UC[26].

Schicho *et al*[23] expanded their findings in DSS mice by studying human subjects with IBD. Their study showed an increase in mannitol, allantoin, xylose, and carnitine in the urine and a decrease in urinary betaine and hippurate during IBD. However, the same as above mentioned studies[25,26], they could not differentiate CD

and UC based on their metabolomics profile[7].

Putting together, based on the metabolomics studies in IBD, the absolute urinary metabolomics signature of IBD is not yet clear. However, the body of literature supports the diagnostic role of urinary metabolites in IBD. More specifically, it seems that microbiota derivative metabolites are altered in IBD and are involved in the pathophysiology of this chronic inflammatory condition. Future multicenter studies on larger sample sizes and with considering confounders such as age, gender and medications should clarify whether urinary metabolomics could be used to: (1) Differentiate UC from CD; (2) predict outcome of the treatments; and (3) define the stage and severity of inflammation.

URINARY METABOLOMICS IN GI CANCERS

GI cancers are common and their burden is huge. Based on a global study in 2013, colorectal, stomach and esophageal cancer are ranked third, fifth and ninth for cancer incidence and fourth, second and sixth for cancer deaths, respectively[27].

Despite available screening method for colorectal cancer which are usually costly and invasive, screening tests for upper GI cancers have not been well developed. Early detection of cancer or pre-cancerous lesions is always desirable. This could benefit from a urine-based cost-effective diagnosis and noninvasive screening assay whereby patients with undiagnosed cancer could be screened.

By analyzing urine samples from esophageal cancer patients and a control healthy group, Hasim *et al*[28] showed that mannitol, glutamate, γ-propalanine, phenylalanine, acetate, allantoin, pyruvate, tyrosine, β-glucose and guinolinate were higher in the urine of patients with esophageal cancer; however, N-acetylcysteine, valine, dihydrothymine, hippurate, methylguanidine, 1-methylnicotin- amide and citric acid were lower. Based on this study, urinary metabolomics could differentiate cancer and control groups. In addition, different pattern of metabolites were positively correlated with the rate of lymph node metastasis and clinical stages. Moreover, unsaturated lipids were a unique marker in differentiating late stages (> 1b2) and early stage (≤ 1b2) diseases[28].

Based on another study, urinary metabolomics signatures clearly distinguished both Barrett's esophagus and esophageal cancer from controls. Although some overlaps were detected, the metabolomics profile of esophageal cancer was different than Barrett's esophagus[29].

Metabolomics studies in gastric cancer are also promising. In a model of gastric adenocarcinoma-bearing mice, the urinary levels of TMAO and hippurate were significantly decreased, although the levels of 3-indoxylsulfate, 2-oxoglutarate, and citrate were significantly increased[30].

Another animal study of implanted human gastric cancer detected significant metabolic differences among normal, non-metastatic and metastatic groups. Based on this study, 10 selected metabolites were different between cancer and control groups. Briefly, the level of lactic acid, butanedioic acid, malic acid, citric acid and uric acid were higher in cancer indicating increase in aerobic glycolysis, respiration (mainly TCA cycle) and the impairment of mitochondrial enzymes. Moreover, glycerol and hexadecanoic acid as indicators of adipocyte lipolysis were higher in cancerous animals. Seven metabolites were also different between non-metastasis and metastasis groups. Alanine and glycerol (as substrates for glycolytic pathway) and L-proline were lower in cancerous animals with metastasis possibly due to a higher level of consumption. On the other hand, the level of myo-inositol in the urine of metastasis group was higher[31].

In a recently published article, the urinary metabolomics of gastric cancer patients was compared to healthy individuals. Based on this study, urinary metabolomics related to amino acids and lipid metabolism was significantly different in cancer *vs* control and could successfully discriminate both groups. Interestingly, the metabolomics signature of cancer showed much higher sensitivity compared to carbohydrate antigen 19-9 and carcinoembryonic antigen. 4-hydroxyphenylacetate, alanine, phenylacetylglycine, mannitol, glycolate, and arginine levels were significantly correlated with cancer T stage. Together with hypoxanthine level, the above mentioned metabolites were tended toward control after surgical treatment[32].

In a study by Chen *et al*[33], urinary lactic acid, arginine, leucine, isoleucine and valine were significantly higher, while citric acid, histidine, methionine, serine, aspartate, malic acid, and succinate were remarkably lower in the gastric cancer patients *vs* controls. In addition, the urinary valine and isoleucine levels were lower in advanced stages compared to early-stages of cancer[33].

Another study also showed that urinary metabolomics could effectively differentiate gastric cancer patients from controls[34]; however, the metabolites which were distinctive, were different than previously mentioned studies[32,33], suggesting complexity in interpreting metabolomics results.

A study on urine metabolites of a colorectal cancer group of patients and their age-matched healthy controls as well as a rat model of chemically induced precancerous colorectal lesion revealed good separations between cancer patients or rats with pre-cancerous lesions and their healthy equivalents. Moreover, altered TCA cycle as well as gut microflora metabolisms were detected in cancer patients and the rat disease model. After surgery, the urinary metabolomic profile of cancer patients altered significantly compared to the preoperative stage since gut microflora metabolism and TCA cycle were down-regulated. In addition, 5-hydroxytryptophan significantly decreased after surgery suggesting an improvement of the tryptophan metabolism[35].

The findings of the above mentioned study in colorectal

cancer were confirmed in a further study which also showed that a panel of urinary metabolite markers composed of citrate, hippurate, p-cresol, 2-aminobutyrate, myristate, putrescine, and kynurenate was able to discriminate colorectal cancer subjects from their healthy counterparts[36].

Studies on the urinary metabolomics of GI cancers reveal alterations in microbiota, proteins and lipid mediated metabolites which are involved in the initiation and dissemination of cancer as well as the cellular overgrowth and proliferation, although no unique signature has been yet recognized. As a huge amount of variability is attributed to between-individual differences, future studies on larger sample sizes of GI cancer patients are required in order to detect associations with moderate effect sizes[37].

URINARY METABOLOMICS IN OTHER GI CONDITIONS

Although many of the metabolomics studies have focused on GI conditions such as cancer and IBD, a few studies have assessed the roles of urinary metabolomics in other diseases.

Based on a study which compared the urinary metabolomics of 34 patients with celiac disease and 34 healthy controls, patients with celiac disease had a significantly lower levels of mannitol, glutamate, glutamine and pyrimidines, and higher levels of indoxyl sulfate, choline, glycine, acetoacetate, uracil, meta-hydroxyphenyl propionic acid, and phenylacetylglycine. This metabolomic signature is consistent with the hypothesis of small bowel dysbiosis in these patients[38]. A further study hypothesized that the metabolomic signature of patients with potential celiac disease, defined as patients with the immunological abnormalities of celiac disease who lack jejunal biopsy findings consistent with their disease, is similar to those with overt celiac disease. Surprisingly, although these patients shared similar metabolomic profile in their serum, no clear joined signature was found in their urine, suggesting that defective small intestinal histology is needed for the development of a urinary metabolomic fingerprint of celiac disease[39].

Studies on the urinary metabolomics of other GI diseases are limited. An animal study has shown the value of urinary metabolomics in the assessment of NSAIDs induced GI ulcer. Based on this study, a panel of urinary metabolites including 2-oxoglutarate, acetate, taurine and hippurate were significant biomarkers for the gastric damage induced by indomethacin in rats and could successfully predict the degree of GI damage, suggesting that NSAIDs induced gastric damage can be possibly screened in the preclinical stages by using urinary metabolomics[40].

CONCLUSION

Urinary metabolomics studies show altered signature in patients with GI disorders compared to healthy controls. The body of literature in this area has majorly focused on IBD and GI cancers. What is shared in all of these disorders is the alteration of urinary metabolites which are in association with GI microbiota and possibly dysbiosis in these chronic conditions. In addition, in cancer patients, the metabolomes which define cell proliferation and differentiation are altered. In IBD, differentiating UC and CD based on urinary metabolomic profile does not look simple at this stage, since confounders such as the clinical severity of the disease and medications may interfere with the metabolism in the body and the metabolomics profile of these patients. The most important use of urinary metabolomics in GI cancer is for early detection of pre-cancerous lesions. Whether the metabolomics signature in patients with pre-cancerous lesions such as Barret's esophagus and colon polyps can predict the future outcome, *i.e.*, the possible chance of progressing to cancer is still under debate. Predicting the outcome of the diseases in response to medical or surgical therapies is also important in this area. In conclusion, although literature supports the role of urinary metabolomics in the diagnosis of some GI conditions, the fingerprints of these diseases are not unique and usually have overlaps.

LIMITATIONS OF URINARY METABOLOMICS IN GI DISORDERS

In 2009, Scalbert *et al*[41] extensively reviewed the limitations of mass-spectrometry-based metabolomics studies. Confounding effects of the diet, large Inter- and intra-individual variations, variations induced by sample collection, handling and storage and inconsistency in data extraction, interpretation and analytical methods were proposed as the major limitations of metabolomics studies. These limitations still affect the metabolomics studies. Moreover, the technology used for the measurement of metabolomics has limitations. For example, NMR is able to measure approximately 8% and gas chromatography MS is able to measure approximately 7% of the human urine metabolomes[4]. For the urinary metabolomics, effects of the kidney function as well as the metabolic function of the body which may affect secretion and reabsorption of the circulating metabolites may confound the final results[42].

FUTURE DIRECTION

Both organic and functional GI disorders usually lack well-defined noninvasive biomarkers which can help us with the diagnosis, treatment and the prediction of their outcome. In functional disorders like irritable bowel syndrome, the diagnosis is not usually definite and is based on exclusion. Moreover, the diagnosis of organic GI disorders usually relies on invasive techniques. Although, the urinary metabolomics signature shows alterations in different GI conditions compared to healthy subjects,

no unique signature has been yet defined. IBD, GI cancers and celiac disease have all shown alterations in the urinary metabolomics which are associated with possible GI dysbiosis, but to our knowledge, no study has systematically evaluated the GI microbiota profile concurrently. Studies on the urinary metabolomics profile of GI diseases have not usually considered confounding factors and the ways of analysis which have been used in these studies are not similar and sometimes cause different results in a single disease setting. Future studies should focus on the validation of the methods and should enhance our knowledge of metabolomic profiles which are in association with different metabolic pathways. The same as breath testing for helicobacter pylori and small bowel bacterial overgrowth, future urinary metabolomics studies may focus on metabolomic profiles induced through the consumption of labeled specific agents. Metabolomics of volatile *vs* non-volatile compounds is also an important area which should be considered. In addition, the effects of urinary diseases on GI system and microbiota as what has been recently observed in patients with chronic kidney diseases[42] should be taken into account when interpreting urinary metabolomics studies.

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Non-surgical factors influencing lymph node yield in colon cancer

Patrick Wood, Colin Peirce, Jurgen Mulsow

Patrick Wood, Colin Peirce, Jurgen Mulsow, Department of Colorectal Surgery, Mater Misericordiae University Hospital, Dublin 7, Ireland

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Correspondence to: Jurgen Mulsow, Consultant Colorectal Surgeon, Department of Colorectal Surgery, Mater Misericordiae University Hospital, Eccles Street, Dublin 7, Ireland. jmulsow@mater.ie
Telephone: +353-1-8545091
Fax: +353-1-8034023

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Abstract

There are numerous factors which can affect the lymph node (LN) yield in colon cancer specimens. The aim of this paper was to identify both modifiable and non-modifiable factors that have been demonstrated to affect colonic resection specimen LN yield and to summarise the pertinent literature on these topics. A literature review of PubMed was performed to identify the potential factors which may influence the LN yield in colon cancer resection specimens. The terms used for the search were: LN, lymphadenectomy, LN yield, LN harvest, LN number, colon cancer and colorectal cancer. Both non-modifiable and modifiable factors were identified. The review identified fifteen non-surgical factors: (13 non-modifiable, 2 modifiable) which may influence LN yield. LN yield is frequently reduced in older, obese patients and those with male sex and increased in patients with right sided, large, and poorly differentiated tumours. Patient ethnicity and lower socioeconomic class may negatively influence LN yield. Pre-operative tumour tattooing appears to increase LN yield. There are many factors that potentially influence the LN yield, although the strength of the association between the two varies greatly. Perfecting oncological resection and pathological analysis remain the cornerstones to achieving good quality and quantity LN yields in patients with colon cancer.

Key words: Lymph node; Number; Factors; Yield; Colon cancer

Core tip: Surgeons, pathologists and patients alike must appreciate that there are many factors which influence lymph node (LN) yield in resected colon cancer specimens. Clinicians must strive for the perfect oncological operation and pathological analysis. However, clinicians should be aware that despite optimal surgery and pathological analysis, other factors may influence the LN yield following colonic resection for cancer.

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INTRODUCTION

The American Joint Committee on Cancer/Union Internationale Contre le Cancer utilises the TNM system to stage colon cancer. The stage of disease is dependent on the depth of penetration into the intestinal wall (T1-T4), the presence of localized lymph node (LN) metastases (N0-N2) and the presence of distant metastases (M0-M1). This system potentially lends itself to "understaging" of disease since accurate staging is closely linked to both adequate and high quality LN evaluation. Indeed, numerous studies have demonstrated an association between the number of LNs examined and patient survival, with the consistent finding that an increased number of evaluated LNs leads to improved survival[1-9]. Furthermore, the decision to administer adjuvant chemotherapy is highly dependent on the presence or absence of LN metastases: When present, patients are classified as stage Ⅲ and typically receive chemotherapy while those with stage Ⅱ disease and no adverse features routinely undergo surveillance only.

The "gold" standard of at least a 12 LN examination following resection for colon cancer was initially proposed in 1990[10]. The National Institute of Clinical Excellence suggested that when more nodes are examined the tumour is significantly more likely to be classified as node positive. Conversely, when few nodes are examined, there is a substantial risk of understaging[11]. This standard has now been adopted in multiple guidelines for both colon and rectal cancer resection specimen analysis[12-14]. More recently, the analysis of ⩾ 12 LNs has been adopted as a standard quality indicator for colorectal resection specimens in the United States by the National Quality Forum[13,15], the National Comprehensive Cancer Network (NCCN), the American Association of Clinical Oncology and the American College of Surgeons[16-18]. However, the literature is variable on the subject with some groups suggesting that a harvest of 9 LNs is sufficient to stage node negative tumours[19], others agreeing that harvesting more than 12 LNs is adequate for staging colon cancer[4,6,20] and others still suggesting that there is no clear cut-off value and that as many LNs as possible should be harvested and analysed[2,8].

Irrespective of the agreed and accepted LN cut-off, it should be appreciated that multiple factors may be influence nodal yield. Undoubtedly, surgical technique and pathological analysis are the cornerstones for adequate LN examination, however other, principally patient factors may be of relevance and lead to reduced LN yield despite optimal surgery and specimen analysis. This study aimed to review both modifiable and non-modifiable non-surgical factors that have been shown to influence colonic resection specimen LN yield and to summarise the pertinent published literature.

LITERATURE REVIEW

A literature review of PubMed for the period 1991-2015 was performed to identify the potential factors which may influence the LN yield in colon cancer resection specimens. The terms used for the search were: LN, lymphadenectomy, LN yield, LN harvest, LN number, colon cancer and colorectal cancer. Both non-modifiable and modifiable factors were identified (Table 1) and the individual papers reviewed. Further relevant publications were identified by cross reference of the reviewed papers.

NON-MODIFIABLE FACTORS

Ethnicity

There have been a number of studies which have assessed the influence of ethnicity on LN yield. The rationale as to why ethnicity would potentially affect the LN yield remains unclear.

In a large study Cone *et al*[21] interrogated the Surveillance Epidemiology and End Results (SEER)-Medicare database in the United States, evaluating all colonic cancer resections between the years 2000 and 2003. Their analysis included nearly 33000 patients, 62.5% of whom had less than 12 LNs in the resected specimen. Multivariate analysis showed that Hispanics were less likely than Caucasian patients to have ⩾ 12 LNs resected (OR = 0.61, 95%CI: 0.5-0.74). Hispanic patients were younger (although all patients in the analyses were older than 65 years), lived in more populated areas and had a lower income status than their Caucasian counterparts. The reduced LN yield did not confer a negative outcome with no significant difference in survival between the groups.

Other smaller single institution studies such as that by Valsecchi *et al*[22] failed to show an association between LN yield and ethnicity.

Age

Numerous studies have assessed the influence of patient age on LN yield in resected colon cancer, the hypothesis being that younger patients are more likely to have a more aggressive oncological procedure, or conversely that older patients frequently undergo less aggressive lymphadenectomy. A national United Kingdom study from 2006 reported that increasing age was associated with a significant reduction in the number of harvested LNs ($P < 0.001$)[23]. Moreover, for every 10 years increase in age in their cohort, there was an associated reduction in LN harvest by 0.9 nodes (95%CI: 0.7-1.1). The authors also noted that as the patient age increased there was also a significant increase in variability of LN harvest between the 79 participating centres ($P < 0.001$), which included both peripheral and tertiary referral centres. These findings were felt most likely to be due to a wider lymphadenectomy being performed in younger, medically fitter (and elective) patients as opposed to older patients with co-morbidities. An alternative

Table 1 Factors influencing lymph node yield in colon cancer resection specimens

Non-modifiable	Modifiable
Ethnicity	Tumour tattooing
Age	Neoadjuvant therapy
Gender	
Socioeconomic class	
Tumour location	
Tumour size	
Tumour histological subtype	
ASA grade	
Tumour classification and stage	
Tumour microsatellite instability	
Lymph node positivity/negativity	
Lymphovascular invasion	
Body mass index	

ASA: American Society of Anesthesiologists.

hypothesis is that LNs undergo a process of involution with increasing age[23].

Stocchi *et al*[24], in their study from the Cleveland Clinic, Ohio, also reported an association in 901 patients with stage Ⅱ colon cancer between increasing age and fewer examined LNs ($P < 0.001$)[24]. Patients younger than 65 years had a mean of 35.1 (range 15-44) nodes examined compared to a mean of 22.2 (range 12-28) nodes in patients older than 65 years.

Another population-based study by Chou *et al*[15] analysed 127927 patients who underwent resection for stages Ⅰ-Ⅲ colon cancer between 1994 and 2005 in the United States. Of note, in 4.6% of patients, no regional LNs were examined and thus, these individual patients were not staged. Once again, age was shown to be a consistently important determinant of LN yield and for every 10-year incremental increase in patient age, there was an associated average reduction of 9% in the number of harvested LNs ($P < 0.01$). It should be noted that over the timeframe of the study, there was not only an increase in the average LN yield, but also a decrease in the mean age at diagnosis for patients with colon cancer - from 70.3 years in 1994 to 68.8 years in 2005. The rationale for this significant association between age and LN yield in this paper was thought to be as a result of a "complex interplay of patient and surgeon factors". The authors explained this by hypothesising that older patients are likely to be considered higher-risk operative candidates and thus a suboptimal surgical dissection (with resultant inadequate lymphadenectomy) may be performed with a view to reducing operative time. Nathan and colleagues also interrogated the SEER database for patients operated with curative intent for stage Ⅰ-Ⅲ colon cancer between 1998 and 2005[25]. In the 27101 patients analysed, increasing patient age was again significantly associated with a decreased LN yield ($P < 0.001$). Finally, Baxter *et al*[26] also interrogated the SEER database, specifically focusing on patient who had undergone colonic resection for pT3 lesions. They identified 11044 patients and once again were able to show that older patients had fewer LNs examined ($P < 0.001$). Several other studies have mirrored these findings[22,27-34], however, 2 smaller studies of 341 and 223 patients respectively, failed to demonstrate an association between colonic LN yield and patient age[35,36].

Overall, it appears that increased patient age significantly influences LN yield in colon cancer.

Patient gender

A number of studies have shown a significant association between male sex and reduced LN yield[25,28,36]. The largest of these included over three hundred thousand patients in a United States population based study analysing factors influencing LN yield in patients with gastrointestinal cancer[28]. The reasons underlying this association are poorly understood. Dubecz *et al*[28] suggested that men are more likely to be uninsured and thus may be less likely to receive "state-of-the-art" treatment which might include adequate lymphadenectomy as performed in a high-volume colorectal centre.

Socioeconomic class

It has been suggested that patients with lower socioeconomic class may be less likely to be treated in specialised centres and to receive the most up to date management with the result that their LN yield following resection for colon cancer is lower. A population-based analysis of all patients with gastrointestinal (GI) adenocarcinomas treated surgically in the United States between 1998 and 2009 ($n = 326243$) was performed by Dubecz *et al*[28]. They aimed to evaluate time trends in lymphadenectomy for GI cancer and to identify factors associated with inadequate LN yield. Adequate lymphadenectomy was defined by the NCCN recommendations as a LN yield of > 12 in colon and rectal cancer. Throughout the study period it was found that the LN yield increased over time for all of the sub classifications of GI cancer. The median number of LNs retrieved for colon cancer increased from 9 in 1998 to 16 in 2009. However, only 49% of patients with a GI adenocarcinoma diagnosis underwent adequate lymphadenectomy. The rate of adequate evaluation was higher in colon cancer (77%) than in rectal cancer (42%). Patients living in areas with higher poverty rates were more likely to undergo inadequate lymphadenectomy. The socioeconomic data was based on county of residence which was linked to United States Census data. The first quartile, Q1 (most well off), had an adequate lymphadenectomy in 49% of cases, Q2 in 51% adequate while Q3 and Q4 (least well off) had adequate lymphadenectomy in 47% of cases. Patients of lower socioeconomic class were most likely to have an inadequate lymphadenectomy with the authors postulating that this finding most likely reflected a high proportion of uninsured patients who may be less likely to receive state of the art treatment. In a separate study, Rajput *et al*[29], also showed that insurance status was associated with LN yield across patients identified from the NCCN and SEER databases. On multivariate analysis, patients with Medicare and Medicaid plans had lower yields than patients covered by commercial plans ($P = 0.007$). The authors' belief was that this finding

was secondary to the age profile of the patients, with those in the Medicare population tending to be older than those with private insurance coupled with a demonstrable decrease in LN yield with increasing age across all patients in the study.

In summary, lower SE status may be associated with reduced LN yield following resection for colon cancer, however there are multiple factors that may underlie this association.

Tumour location

There is consistent evidence that the location within the colon of the primary is strongly associated with the number of LN examined by the pathologist, with the length of the specimen often implicated as the causative factor. Stocchi *et al*[24] reported that a 12 LN harvest was more likely with right sided as opposed to left sided carcinomas (85% *vs* 72%, $P < 0.001$). Similarly, in the study from Baxter *et al*[26], patients with a left sided colon cancer (and rectal cancer) were less likely to have an adequate LN evaluation compared with patients with right sided lesions. In a separate study, Wright *et al*[37] reported a median number of 12 LNs for right sided cancer and 9 LNs for left sided colonic tumours. Chou *et al*[15] also reported a similar trend: In a sub-analysis of right and left sided colon cancers, tumours located in the ascending colon and hepatic flexure had, on average, 34% more LNs retrieved than those in the sigmoid and rectosigmoid. However, the authors acknowledged that the SEER data on which their study was based did not record the length of the resected specimen and speculated that the observed differences in LN yield may in fact be due to longer specimen lengths following right sided resection. The association between specimen length and LN yield has been repeatedly demonstrated. Stocchi *et al*[24] showed that specimens less than 30 cm in length had a median LN harvest of 17 nodes whereas those longer than 30 cm had a median harvest of 24 nodes ($P < 0.001$)[24]. Shen *et al*[31] also reported variability in LN yield depending on both tumour site and specimen length. They studied 365 resected colon cancers and demonstrated an increased LN yield of 17.8 for caecal and ascending colon lesions *vs* 14.3 for sigmoid lesions ($P < 0.01$). Descending colon lesions were associated with the longest specimens at 29.2 cm and there was a clear association between the length of the specimen and LN yield, with an average of 11 LNs in specimens of 10 cm or less in length compared with 18.3 LNs when the specimens were over 30 cm in length. Numerous studies have shown similar patterns of decreased LN yield for left-sided *vs* right-sided colonic cancer[22,27,29,33,34,38-40]. Two smaller studies, analysing 137 and 48 colon specimens respectively, failed to show an association between LN yield and primary tumour site[41,42].

In summary, the published literature supports the hypothesis that tumour location influences LN yield in colon cancer.

Tumour size

It has previously been proposed that larger tumours elicit an intense antigenic response within the surrounding regional LN basin. This "response" may potentially make them more visible to pathologic examination and may thus lead to an increased LN yield[37]. In a study by Chou *et al*[15], for every 1 cm increase in tumour size, there was a corresponding average 2% increase in the number of examined LNs in colon cancer specimens. Tumour size was also shown to be a significant predictor of LN yield in univariate analysis in a study by Valsecchi *et al*[22] ($P < 0.01$). There have been 2 recent studies from the Memorial Sloan Kettering group, both reporting a strong association between tumour size and the nodal yield[27,43]. In the first study, tumour size of 4 cm or less resulted in a mean nodal harvest of 19.7 as compared to a mean nodal harvest of 23.3 when the tumour measured over 4 cm ($P = 0.02$)[27]. In the second and more recent study, analysis of 256 colectomy specimens demonstrated a linear relationship between tumour size and LN yield ($P < 0.0001$)[43]. Søreide *et al*[39] also showed that LN harvest is related to tumour size. Tumours greater than 5 cm had adequate LN yield in 50% of cases, compared to 24%, when tumour size were less than 5 cm.

Colon cancer histological subtype and tumour differentiation

Tekkis *et al*[23], in a study including more than 5000 patients, showed that the tumour differentiation was one of eight factors which had a significant influence on the number of LNs examined. Poorly differentiated tumours had significantly increased LN yield when compared to well or moderately differentiated lesions. In the same study, the tumour subtype was not shown to significantly influence nodal yield.

A number of other studies have reported an association between tumour differentiation subtype and LN yield, with the consensus being that the more poorly differentiated the tumour the greater the LN yield compared to well differentiated lesions[25,27,35,37].

ASA grade

The evidence to support an association between American Society of Anesthesiologists (ASA) grade and LN yield is limited. The rationale behind linking ASA grade and LN yield is similar to that for increasing age. Patients with higher ASA are often older and may undergo emergent surgery, which may lead to less radical dissection in order to complete the operation in a timelier manner. A national United Kingdom study published in 2004[23] did show that patients with higher ASA grade were less likely to have adequate LN harvesting when compared to patients with lower ASA grades: ASA Ⅲ *vs* Ⅰ ($P < 0.001$) and ASA Ⅳ-Ⅴ *vs* Ⅰ ($P = 0.036$).

LN positivity

The available literature shows conflicting findings with respect to the influence of LN positivity on LN yield. Any

association, positive or otherwise, should be interpreted with some caution due to the potential for underlying bias. An association between increased LN yield and nodal positivity, as shown by Tekkis *et al*[23] for example, may simply reflect a more comprehension search for nodes. On the other hand, a finding of multiple involved nodes may lead to a less thorough search for further nodes leading to a lower overall nodal yield. In a study by Nash *et al*[27], no correlation was demonstrable between the total number of LNs examined and the number of LNs with metastatic disease ($P = 0.32$). However, there was a trend towards finding one fewer LN in each specimen for every 2 metastatic LNs.

Lymphovascular invasion

Lymphovascular invasion (LVI) is a surrogate marker for tumour aggressiveness and is associated with a poorer outcome. The limited available data shows no association between LVI and LN yield. Gelos *et al*[35] performed a retrospective analysis of 341 patients who underwent colorectal cancer resection with curative intent between 2000 and 2005 and investigated the impact of a number of factors including LVI on LN yield. There was a median of 15.17 LNs retrieved per patient, with 82.8% of the 341 patients having a LN harvest greater than 12, however the presence of LVI did not influence tumour LN yield. In another smaller study (48 patients) with a mean LN count of 14.1, no statistically significant relationship existed between the number of LNs and the presence of LVI ($P = 0.64$)[42].

Microsatellite instability

An association between LN yield and microsatellite instability (MSI) has been put forward by a number of authors. MSI tumours are considered less aggressive than their microsatellite stable (MSS) counterparts and may demonstrate an enhanced host inflammatory reaction[44-47].

An association between a high rate of MSI and a high total LN count in colorectal cancer has been demonstrated in a number of small studies. Higher LN retrieval may in part explain the improved survival seen in patients with MSI. Søreide *et al*[39] studied 121 patients under the age of 75 with the aim of determining whether proximal tumour location and MSI improved LN yield. One thousand two hundred (1200) LNs were retrieved from 121 patients and of these, 96 were positive (0.8%). Median LN harvest was 10 and only 36% of patients had an adequate harvest (*i.e.*, 12 or more LN). MSI was found in 33 out of the 121 patients (27%) and this was associated with a greater median LN yield of 12 *vs* 9 in the MSS group. Fifty-four percent of patients with MSI had adequate LN harvest *vs* 29% in the MSS group and 36% in the study as a whole [OR = 2.9 (1.3-6.5), $P = 0.011$][39].

Eveno *et al*[48] reported a smaller series of 82 patients with stages Ⅰ and Ⅱ colon cancer and also showed a significantly increased LN yield in the MSI group (mean 23.6 *vs* 13.7 LN).

A separate study investigated the association between MSI and LN yield but did not show a significant association[49]. Of 168 patients with stage Ⅲ colon cancer the mean total LN yield for MSI and MSS tumours was 15.9 and 16.9 respectively ($P = 0.664$). The authors concluded that increased survival in the MSI group ($P = 0.026$) could not be explained by differences in LN yield.

Body mass index

Studies performed in patients with gastric and rectal cancers have shown an association between obesity and reduced LN yield[50,51]. Damadi *et al*[52] retrospectively reviewed 191 patients who underwent a resection for colon cancer between 1999-2006. They hypothesized that obese patients with a body mass index (BMI) > 30 kg/m^2 would have a smaller yield of LNs compared to non-obese patients with a BMI < 30 kg/m^2, however they found no significant difference between the groups (mean LN yield 12.7 in obese *vs* 12.4 in non-obese, $P > 0.2$).

Linebarger *et al*[53] performed a retrospective review of 401 patients, and stratified them into six groups based on BMI: Underweight (< 18.5 kg/m^2), normal (18.5-24.9 kg/m^2), overweight (25-29.9 kg/m^2), stage Ⅰ obesity (30-34.9 kg/m^2), stage Ⅱ obesity (35-39.9 kg/m^2) and stage Ⅲ obesity (> 40 kg/m^2). They found no significant difference in the number of LNs harvested for each of the groups.

Kuo *et al*[54] retrospectively analysed 645 patients with stage Ⅲ colon cancer from Taiwan who underwent colectomy. Patients were again placed into four groups based on their BMI: Obese (BMI > 27 kg/m^2), overweight (BMI 24-27 kg/m^2), normal (BMI 18.5-24 kg/m^2) and underweight (BMI < 18.5 kg/m^2). The mean BMI of the patients in the study was 23 kg/m^2. The authors showed a significantly increased mean LN yield in the underweight patient group (28.1 *vs* 23 in the normal BMI group, 19.5 in the overweight group and 19.8 in obese patient group respectively). A 2010 study analysed a cohort of 718 NCCN patients with stage Ⅰ-Ⅲ colon cancer and found three factors were associated with not meeting the quality standard of a 12 LN evaluation: Left-sided tumours, stage Ⅰ disease and a BMI > 30 kg/m^2[28].

The impact of BMI on LN yield is overall unclear, with some studies pointing to a reduced in patients with a higher BMI.

MODIFIABLE FACTORS

Tumour tattooing

Endoscopic tattooing is frequently performed in order to facilitate tumour localisation during laparoscopic resection. Tumour tattooing may inadvertently map the sentinel node and associated draining nodes and thus make them more readily identifiable for pathological evaluation. A retrospective case controlled trial conducted between 2005 and 2009 aimed to determine if colonoscopic tumour tattooing could be utilised to increase staging accuracy by increasing the LN yield[55]. The

authors assessed two groups of patients: The first group contained a series of 95 consecutively tattooed patients and the second group a series of 210 non-tattooed patients. All patients underwent surgery for colorectal cancer within the same time period. There was a higher LN yield in patients with pre-operative tattooing compared to the non-tattooed control group (median LN yield of 15 *vs* 12 nodes, $P = 0.014$). Multivariate analysis showed that the presence of carbon-containing LNs (with a detection rate of 71%) was an independent predictor for an increased LN yield ($P = 0.002$), although the reason for the lack of a predictive characteristic for the group in which tattooing did not result in carbon containing LNs was not clear.

The potential role of preoperative tattooing was also reported by Nash *et al*[27]. Their study was designed to develop a predictive model of LN yield in colon cancer. One hundred and fifty-two specimens from patients who had undergone resection for colon cancer were used, with detailed anatomical and surgical technique documentation on each specimen. A linear regression analysis was performed and this identified both predictors and confounders of the quantity of the LN harvest. Of the 15 variables analysed, it was found that tumour size, tumour location, number of resected pedicles and use of pre-operative tattoo had significant linear/quadratic relationships on the LN yield. When controlling for the 14 other variables, patients who underwent endoscopic tattooing had 3.1 more LNs harvested. This data further suggests that endoscopic tattooing may be used pre-operatively to maximise LN yield and increase the accuracy of disease staging. The authors acknowledged that as they did not record the proportion of LNs which harboured grossly apparent dye at the time of LN identification, they could not make a definitive conclusion as to the mechanism by which preoperative colonic cancer tattooing might increase LN yield Dawson *et al*[56] also hypothesised that pre-operative tattooing with India ink might increase the subsequent LN yield from the resected specimens. Their retrospective study included 174 patients who underwent surgery for colon cancer between 2006 and 2009. Sixty-two patients had pre-operative tattooing. The mean number of LNs harvested in the tattooed group was 23 compared to 19 in the non-tattooed group ($P = 0.03$). In the tattooed colon cancer group a 12 LN minimum was achieved in 87.1% patients *vs* 72.3% in the non-tattooed group. These results were mirrored in a separate analysis, within the same study, of 35 patients with rectal cancer. Once again, the results from this study suggest the routine utilisation of pre-operative colonoscopic tattooing may increase the LN yield in resected colonic malignancy.

Neoadjuvant chemotherapy

The role of neoadjuvant therapy in the setting of colon cancer remains in evolution. Data from studies performed in patients with rectal cancer has shown that neoadjuvant therapy may result in a decreased LN yield, however this is in the context of both radiotherapy and chemotherapy. The initial data from the United Kingdom based FOxTROT trial reported on 150 patients in 35 centres[57]. All patients had either T3 (with > 5 mm invasion into the muscularis propria) or T4 colon tumours and were randomised to either preoperative and postoperative chemotherapy or standard postoperative chemotherapy alone (2:1 randomisation). Overall, the authors reported that preoperative chemotherapy was a viable option with acceptable toxicity in this cohort. When the LN data were examined, 85 of 98 patients (87%) and 43 of 50 patients (86%) had 12 or more LNs examined in the combined preoperative and postoperative chemotherapy and postoperative chemotherapy groups respectively. Indeed, 46% and 54% of patients in both groups had greater than 20 LNs examined with median values of 21 and 22 nodes respectively ($P = 0.2$). The apical node was positive in 1 of 98 patients in the combined group (1%) and 10 of 50 patients in the postoperative chemotherapy only group (20%). Thus, in this study neoadjuvant chemotherapy did not result in a lower LN yield however more data is needed before definitive conclusions can be made.

CONCLUSION

There are many factors that can potentially influence the LN yield following resection for colon cancer and the relationship between these factors remains poorly understood. High quality oncological surgery and pathological analysis are the most important factors in ensuring optimal LN yield. However, the current review has highlighted a number of additional modifiable and non-modifiable factors that may also influence the number of LNs harvested. Older age, obesity, and male sex may be associated with reduced LN yield. Similarly, studies have shown an association between ethnicity and lower socioeconomic class and reduced LN harvest. Rather than being true associations, however, it is likely that these findings reflect, at least in part, external modifiable factors such as the surgeon's attitude to older patients undergoing surgery or the quality of care received by patients in lower SE groups. LN yield appears to be increased in patients with right-sided cancer, bulky tumours, or poor tumour differentiation. Again, these associations may reflect other factors known to influence nodal yield such as the length of the resection specimen. Nonetheless, these variables should be taken into consideration when evaluating the completeness of the LN harvest for individual patients.

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ORIGINAL ARTICLE

Retrospective Study

Intensity modulated radiation therapy with simultaneous integrated boost based dose escalation on neoadjuvant chemoradiation therapy for locally advanced distal esophageal adenocarcinoma

Ming Zeng, Fernando N Aguila, Taral Patel, Mark Knapp, Xue-Qiang Zhu, Xi-Lin Chen, Phillip D Price

Ming Zeng, Fernando N Aguila, Taral Patel, Mark Knapp, Xue-Qiang Zhu, Xi-Lin Chen, Phillip D Price, Department of Radiation Oncology, Mount Carmel Health System, Columbus, OH 43219, United States

Ming Zeng, Xue-Qiang Zhu, Cancer Center, Sichuan Academy of Medical Sciences, Sichuan Provincial Hospital, Chengdu 610072, Sichuan Province, China

Fernando N Aguila, Central Ohio Surgical Associates, Inc., Columbus, OH 43219, United States

Taral Patel, Mark Knapp, Department of Hematology and Oncology, Zangmeister Cancer Center, Mount Carmel Health System, Columbus, OH 43219, United States

Xi-Lin Chen, Department of Oncology, 307 Hospital, Beijing 100071, China

Author contributions: Zeng M, Aguila FN, Patel T and Knapp M involved the study protocol design; Zhu XQ and Chen XL collected and analyzed the data; Zeng M, Patel T and Chen X draft the manuscript.

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Correspondence to: Ming Zeng, MD, PhD, Department of Radiation Oncology, Mount Carmel Health System, 3100 Plaza Properties Blvd, Columbus, OH 43219, United States. miller2002@yahoo.com
Telephone: +1-614-2168721

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Abstract

AIM: To evaluate impact of radiation therapy dose escalation through intensity modulated radiation therapy with simultaneous integrated boost (IMRT-SIB).

METHODS: We retrospectively reviewed the patients who underwent four-dimensional-based IMRT-SIB-based neoadjuvant chemoradiation protocol. During the concurrent chemoradiation therapy, radiation therapy was through IMRT-SIB delivered in 28 consecutive daily fractions with total radiation doses of 56 Gy to tumor and 5040 Gy dose-painted to clinical tumor volume, with a regimen at the discretion of the treating medical oncologist. This was followed by surgical tumor resection. We analyzed pathological completion response (pCR) rates its relationship with overall survival and event-free

survival.

RESULTS: Seventeen patients underwent dose escalation with the IMRT-SIB protocol between 2007 and 2014 and their records were available for analysis. Among the IMRT-SIB-treated patients, the toxicity appeared mild, the most common side effects were grade 1-3 esophagitis (46%) and pneumonitis (11.7%). There were no cardiac events. The Ro resection rate was 94% (n = 16), the pCR rate was 47% (n = 8), and the postoperative morbidity was zero. There was one mediastinal failure found, one patient had local failure at the anastomosis site, and the majority of failures were distant in the lung or bone. The 3-year disease-free survival and overall survival rates were 41% (n = 7) and 53% (n = 9), respectively.

CONCLUSION: The dose escalation through IMRT-SIB in the chemoradiation regimen seems responsible for down-staging the distal esophageal with well-tolerated complications.

Key words: Intensity modulated radiation therapy; Esophageal adenocarcinoma; Simultaneous integrated boost; Neoadjuvant chemoradiation; Dose escalation; Resection rate

Core tip: There are more data supporting neoadjuvant chemoradiation for locally advanced esophageal cancer. The best regimen of neoadjuvant chemoradiation remains to be defined, current available data using three-dimensional *vs* intensity modulated radiation therapy deliver modest dose to downstage the tumor. In this report, we reviewed our experience using dose escalation technique to Gross Tumor Volume with compromising dose to organ at risk, the high R0 resection rate results suggest the feasibility of using this approach for future prospective study.

Zeng M, Aguila FN, Patel T, Knapp M, Zhu XQ, Chen XL, Price PD. Intensity modulated radiation therapy with simultaneous integrated boost based dose escalation on neoadjuvant chemoradiation therapy for locally advanced distal esophageal adenocarcinoma. *World J Gastrointest Oncol* 2016; 8(5): 474-480 Available from: URL: http://www.wjgnet.com/1948-5204/full/v8/i5/474.htm DOI: http://dx.doi.org/10.4251/wjgo.v8.i5.474

INTRODUCTION

Distal esophageal cancer is a commonly lethal malignancy, with the annual death rate in the United States about 15590[1]. Most single-modality treatment regimens provide poor cure rate. For example, surgical treatment alone has a 5-year survival of approximately 15% to 20%[2,3]. Radiation therapy alone for resectable highly selected squamous cell cancer results in a 5-year survival rate of approximately 34%[4]. In recent decades, more data have emerged for locally regional esophageal cancer management. There are reports that suggest triple-modality treatment regimens provide better local control, better control of distant metastases, and better overall survival[5]. Meta-analysis showed a survival benefit for patients treated with preoperative chemoradiotherapy (CRT) compared with surgery alone[6]. We have been using triple-modality approach for locally advanced distal esophageal cancer. Although the results from CROSS studies demonstrated the triple-modality approach to be well-tolerated with survival benefit, the absolute benefit for adenocarcinoma remains small and the survival benefit for adenocarcinoma is not statistically significant[7], with a 17% pathological complete response (pCR). There is clear evidence correlating pCR and better local control with improved survival[8]. However, there are few reports regarding the role of radiation dose variation in triple-modality therapy. Understanding dose change and its impact on tumor response provides insight into the effectiveness of the combined treatment and may lead to improved survival. Therefore, we analyzed our experience with dose escalation through the intensity modulated radiation therapy with simultaneous integrated boost (IMRT-SIB) technique, with emphasis on the relationship of the site of pCR to the radiation dose escalation delivered to the gross tumor volume.

MATERIALS AND METHODS

Our Institutional Review Board approved this study. The patients included in this study were treated at our institution for locally advanced esophageal cancer between January 2007 and December 2014. The inclusion criteria were pathologically confirmed adenocarcinoma and no past or current history of malignancy or radiation treatment in the chest or abdomen. Written informed consent was obtained from all patients before starting treatment. All patients underwent staging and those with no distant disease and medically operable patients received neoadjuvant CRT for 5-6 wk. The resectable disease included stage cT1N1M0 or cT2-3N0-1M0 (Table 1)[9].

Pretreatment staging included elaborate history taking, physical examination, routine blood studies, pulmonary function tests, an upper gastrointestinal endoscopy with biopsies, and computed tomography (CT) of chest, abdomen and neck. Endoscopic ultrasound was used routinely for staging of esophageal tumors if technically possible. All patients in this analysis underwent positron emission tomographic (PET) scanning as part of staging to better define gross tumor volume.

All treatment began with concurrent chemoradiation. Chemotherapy consisted of five cycles of concurrent platin-based weekly chemotherapy or 5-fluorouracil (5-FU)-based daily chemotherapy. The platin-based chemotherapy was given at a dose of 40-100 mg/m^2, starting on days 1, 8, 15, 22 and 29. The 5-FU-based chemotherapy was either continuous infusion CIV 5-FU (225 mg/m^2) or oral capecitabine (capecitabine,

Table 1 Characteristics of 17 patients

Characteristic	Value
Mean age (range)	65 yr (45-76)
Men/women (*n*)	14/3
ECOG PS (*n*)	
0	14
1	3
2	0
3	0
T stage (*n*)	
T1	0
T2	2
T3	15
T4	0
N stage (*n*)	
N0	12
N1	5
Concurrent chemotherapy (*n*)	
5-FU/cisplatin	11
Carboplatin/Taxel	6
Tumor location: < 35 cm/> 35 cm (*n*)	14/3
Mean gross tumor volume (cm^3) (range)	43.7 (33.3-62.4)
Mean clinical tumor volume (cm^3) (range)	503.2 (419.3-577.1)

ECOG PS: Eastern Cooperative Oncology Group performance status; 5-FU: 5-fluorouracil.

Genentech, San Francisco, CA) 750 mg/m^2 twice daily starting on day 1 to day 28.

The radiation therapy could be delivered through four-dimensional (4D) plus IMRT with SIB. All patients who received radiation therapy started with CT-based 4D treatment simulations. The simulation was performed with the patient in the supine position using immobilization with the patient's arms over the head. The 4D simulations were performed if respiratory gating was feasible, otherwise, free-breathing 3D CT acquisition data would be obtained during simulation, the patients then were excluded from the IMRT-SIB protocol. All treatment planning in this series was performed by the same radiation oncologist. During the treatment planning, two target volumes were drawn gross tumor volume and clinical target volume, PET/CT imaging obtained within 1-3 wk prior to simulation data. Patients who did not have PET/CT data were excluded from the IMRT-SIB protocol. Clinical target volume and the clinical internal target volume reflected the microscopic sites of highest risk. The treatment planning target volume (PTV) for clinical target volume is about 5 mm beyond clinical target volume; the clinical target volume was contoured based on the Radiation Therapy Oncology Group consensus study protocol[10]. The treatment PTV for gross tumor volume had no margins.

There are total 17 patients received SIB technique and included in this study, after resection the postoperative T stages of the analyzed tumors were as follows: ypT0 (*n* = 8, 47%); ypT1 (*n* = 4, 23.5%); ypT2 (*n* = 3, 17.6%); ypT3 (*n* = 2, 11.7%). Nodal disease was confirmed in three patients (17.6%) by pathological staging and the median number of assessed lymph nodes was 13 (range 3-27) (Table 2). There were five pulmonary complications (29%), one cardiac complication (5.8%), and six surgical complications (35%). There were no treatment-related or operative deaths (Table 3).

Table 2 Pathological staging post simultaneous integrated boost based neoadjuvant chemoradiation

Pathological staging	Patient *n* (%)
ypT	
0	8 (47)
1	4 (23)
2	3 (18)
3	2 (12)
4	0
ypN	
0	13 (76.5)
1	4 (23.5)

The time required to finish radiation treatment ranged from 28-35 d. Clinical tumor volume (CTV) represents the conventional dose coverage, 5040 in 28 fractions and PET-positive alone target area will receive SIB to 5600 in 28 fractions, which is labeled as gross tumor volume (Figure 1). The PTV of 180 cGy per fraction provided a proximal and distal margin of 5 cm and a radial margin of 7 mm around the CTV volume except to the heart with approximately 3-5 mm margins. The average beam number was 6.3 (range, 5-9). All organs at risk met their dose constraints. The daily prescription dose of 2 Gy was specified at the International Commission on Radiation Units and Measurement reference point, and at least the 95% isodose had to encompass the entire PTV. The maximum dose to the PTV was not to exceed the prescription dose by 7%. Tissue density inhomogeneity correction was used. The 4D plan using respiratory gating technique applied to all patients.

Patients were followed routinely after finishing neoadjuvant chemoradiation. Surgical resection was performed between 6-8 wk after completion of CRT. The operative technique consisted of a transthoracic approach with a two-field lymph node dissection or a transhiatal approach, depending on tumor localization. A wide local excision of the N1 lymph nodes, including standard excision of the celiac nodes, was carried out in both techniques. Continuity of the digestive tract was restored by gastric tube reconstruction or colonic interposition procedure with cervical anastomosis.

For grading of the therapy response, the degree of histomorphologic regression was classified into four modified categories, as described by Mandard *et al*[11]. Surgical margins were designated in accordance with the criteria of the AJCC staging manual. All resection margins, including circumferential margins, were evaluated for vital tumor with a cutoff point of 1 mm. Margin status was confirmed by frozen and permanent sections and the close distance to the nearest millimeter between cancer cells, and the margin was measured microscopically and recorded prospectively. The operation was defined as an R0 resection if there was no microscopic tumor found at the margin and as an R1 resection if a margin was positive microscopically.

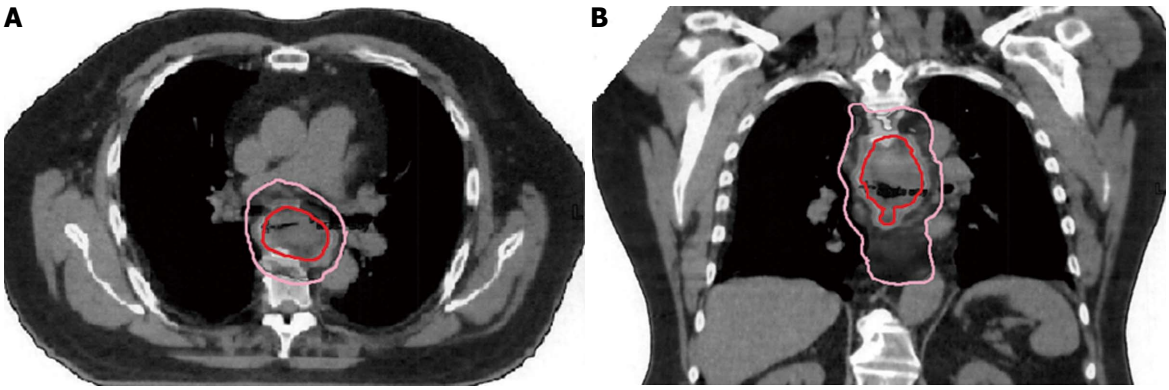

Figure 1 Transverse (A) and coronal (B) images of representative four-dimensional intensity modulated radiation therapy with simultaneous integrated boost. The isodose lines for 5040 cGy (pink) and 5600 cGy (red) were labeled.

Table 3 Postoperative toxicity after neoadjuvant treatment followed by surgery

Pulmonary	5
Pneumonia	3
Pneumonitis	2
Cardiac[1]	1
Surgery related	6
Anastomotic leakage	4
Anastomotic stricture	1
Wound infection	1
Death within admission	0

[1]Cardiac toxicities include heart attack, cardiac rhythm changes, pericardia effusion.

Duration of follow-up was defined as the interval between the day of completed surgery, death, or the last follow-up visit or telephone call. The Kaplan-Meier method was used to calculate survival probabilities. Survival analyses were performed using Prism Graph Pad Version 5.00 (GraphPad Software, Inc., LaJolla, CA).

RESULTS

During the study period, 57 patients underwent neoadjuvant chemoradiation therapy followed by esophagostomy. After exclusion of patients with insufficient data, those who did not meet study criteria, and those without adequate follow-up (n = 22), the records of 35 patients were reviewed. Of the 35, 19 patients were eligible and enrolled in the IMRT-SIB treatment protocol and two patients without histological identification of adenocarcinoma were excluded from this analysis. Thus, 17 patients completed the IMRT-SIB treatment and were included in this study. There were 15 men and 2 women and the mean postoperative follow-up was 2.5 years (range from 0.22-6.5).

All staging was performed before any treatment began. The mean age at time of diagnosis was 65 years (range, 45-76 years) and 15 patients were men. Of all patients, 88% had a uT3 tumor. A microscopically radical (R0) resection was achieved in 94% of patients. One patient had an R1 resection due to persistent disease in the gastric cardia. Total pathologic complete response ypT0No is 47%. The node-negative patient after neoadjuvant chemoRT is 82% (Table 2). Table 2 shows the pathologic staging and effects of SIB-based neoadjuvant CRT. The pathologic stages of cancer were T0, T1, T2, T3, and T4 in 8 (47%), 4 (23%), 3 (18%), 2 (12%), and 0 (0%), respectively. A comparison of clinical and pathologic stages revealed that SIB-based neoadjuvant CRT resulted in down-staging of either the T or the N status of 13 (76.5%) patients.

Hematologic toxicity from neoadjuvant chemoradiation with SIB was mild for all patients. A few patients received granulocyte-colony stimulating factor for neutropenia that did not occur during CRT. Among non-hematologic adverse effects, esophagitis (n = 10, 58.8%) and pneumonia (n = 3, 17%) was more common than pneumonitis (n = 2, 11%). Surgical leak was the most common surgical-related complication (n = 4, 23.5%) (Table 3). No survivors had symptoms due to late toxicities such as accumulated pleural or cardiac effusions during long-term follow-up. There were no treatment-related deaths.

After a minimum follow-up of 22 mo and a mean survival of 29 mo, the local recurrence rate was 11% (n = 2). Most patients had distant failure (35%) or combined local/regional and distant failure (5%). The majority of local recurrences were within 2 years of follow-up. In addition, the anastomosis was the only recurrence site in 5.8% of patients. There was one mediastinal relapse associated with positive nodes that was treated with a full dose of IMRT-SIB. There was no peritoneal carcinomatosis found.

For the 17 patients analyzed, nine were alive and eight had died at the end of follow-up, for a 3-year overall survival rate of 52% (Figure 2). Among the pCR, the 3-year overall survival was 75%, and compared with pathological persistent disease (pPD) the survival was 33% (Figure 2A). The 3-year disease-free survival rate for the 17 patients analyzed was 41.2%. The disease-free survival for the pCR and pPD subgroups were 63.55% and 22.2%, respectively (Figure 2B). There is no statistical significance among the overall survival analysis (P = 0.0523) and disease-free survival analysis (P = 0.0897). However, there is trend toward improved

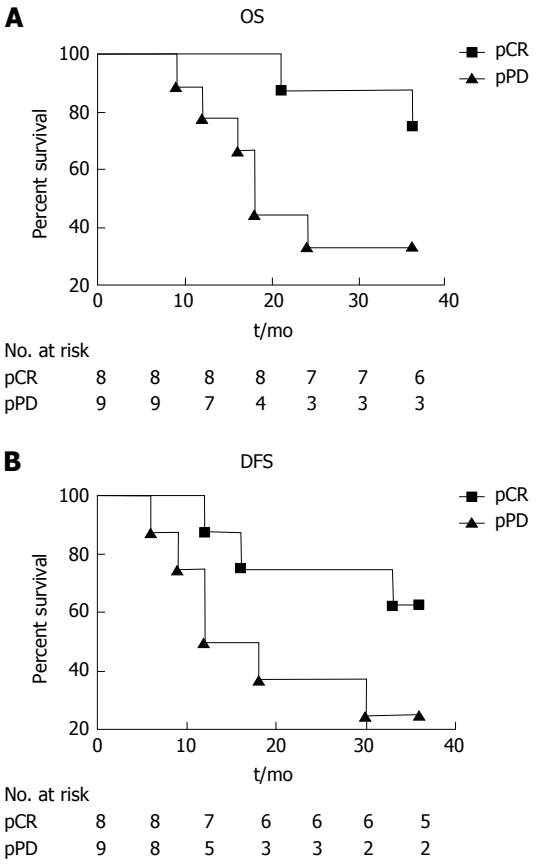

Figure 2 Kaplan-Maier survival curve. A: Overall survival for patients with pCR or without (pPD) (P = 0.523; χ^2 = 3.767); B: Disease-free survival (P = 0.897; χ^2 = 2.879). The disease-free survival represented from both local regional recurrences at anastomotic site, mediastinum, celiac trunk, or supraclavicular lymph nodes and distant metastases. OS: Overall survival; DFS: Disease free survival; pCR: Pathological completion response; pPD: Pathological persistent disease.

prognosis when comparing pCR *vs* not complete response, although this did not reach statistical significance.

In univariate survival analysis, among the various factors, post-triple modality node stages were a strong independent favorable predicting factor for survival (P < 0.01). Sex, the tumor size, the type of chemotherapy, the tumor location, and tumor configuration were not predicting factors for survival (Table 4).

Patients who had pCR to neoadjuvant treatment had a trend of benefit for the probability of survival compared to patients with pPD after neoadjuvant treatment. The 3-year overall survival rates were 75% *vs* 33% and the 3-year disease-free survival rates were 62.5% *vs* 22.2%, respectively. Interestingly, the analysis of histological regression after neoadjuvant showed improved survival rates if no or only rare residual tumor cells were found in the node specimen (Table 4).

DISCUSSION

Many reports show a consistent finding that response to preoperative therapy, particularly the absence of residual disease in the surgical specimen, is a good outcome indicator of better disease-free and overall survival[8,12-14]. In a comprehensive literature review of 22 studies in

Table 4 Survival by prognostic factor

		Overall survival (%)		
		1 yr	3 yr	*P* value
Sex				
Female	2	100 (2)	100 (2)	0.017584
Male	15	73 (11)	47 (7)	
Tumor size				
> 3 cm	9	78 (7)	44 (4)	0.34649
< 3 cm	6	83 (5)	67 (4)	
Unknown	2	50 (1)	50 (1)	
Concurrent chemo				
5-FU based	11	82 (9)	55 (6)	0.078225
Cis based	6	83 (5)	50 (3)	
Tumor configuration				
> 180 cm	8	63 (5)	50 (4)	0.382319
< 180 cm	9	89 (8)	55 (5)	
Node status				
Negative	13	85 (11)	62 (8)	0.009262
Positive	4	75 (3)	25 (1)	
Location of primary tumor				
20-35 cm	12	83 (10)	58 (7)	0.024229
> 35 ~	5	80 (4)	40 (2)	
Total	17	82 (14)	53 (9)	

5-FU: 5-fluorouracil.

which patients with esophageal or esophagogastric junction cancer underwent esophagectomy after neoadjuvant CRT, patients with a pCR were two to three times more likely to survive than were those with residual disease in the esophagectomy specimen[15]. These benefits translate into a 33% to 36% mean absolute survival benefit when a pCR is achieved than when it is not. These assumptions provide the rationale for intensification of preoperative treatment *via* a higher biological dose delivered to the tumor mass, without increasing the surrounding organs' risk of toxicity. The IMRT-SIB approach was based upon better radiographic findings of the biological target through PET scanning prior to CRT. This approach provides a better outline of the biological tumor within a mass, and allows RT dose intensity increase by 10% to the biological activity of tumor, without increasing the overall treatment time and the dose to the surrounding organs at risk[16].

In the Cross study, although the improvement in overall survival is 14%, the improvement for adenocarcinoma is limited[7]. The trend of improvement is not statistically significant. The greatest benefit was for squamous cell cancer. Few data were available on how to improve the outcome for esophageal adenocarcinoma and gastroesophageal cancers. Most reports had histology of adenocarcinoma and squamous cell cancer.

We reviewed our single-institute experience on adenocarcinoma using IMRT-SIB dose escalation technique, and found that the toxicity is low and local and that the incidence of distal recurrence is consistent with previous reports[11,17,18]. Moreover, we found a possible association between that dose escalation with IMRT-SIB and the high pCR rate. The idea behind preoperative CRT in the treatment of esophageal and gastroesophageal junction cancer was to improve survival by reducing

locoregional failure[13,18]. The high pCR rate through IMRT-SIB dose escalation to the tumor mass itself is a strong favorable prognostic factor for both locoregional and systemic recurrence and maybe even for overall survival[19]. However, further studies are needed to explore the potential association between SIB-induced pCR and overall survival rate for adenocarcinoma.

The Cross study[7] and the report by Hoeppner *et al*[20] used less than 4500 cGy dose protocol. In the Cross study, the low dose could explain the less favorable results for adenocarcinoma compared with squamous cell cancer histology[7]. In the Hoeppner *et al*[20] report, the low-dose radiation could explain the less favorable results for neoadjuvant chemoradiation compared with perioperative chemotherapy for adenocarcinoma. At least, the suggestion of the low dose of radiation is one of factor contributed to low pCR was reported[21]. In addition, whether predicting value of the SIB induced pCR and conventional dose resulted pCR are the same? Is there anything else be young dose response relationship between the gross tumor volume and delivered dose? Does dose escalation impact on risk of distant metastasis? More future studies need to explore above questions.

Since all cancers in the present study were in the lower distal esophagus, the radiation field was limited to the lower mediastinum and did not include in supraclavicular regions, and there were no recurrences in the supraclavicular areas. Strict dose constrains to the heart were used and no significant cardiac events were noted. Although some reports suggested that higher treatment morbidity and mortality were associated with neoadjuvant CRT[20,22], the current cohort's patients had acceptable tolerance.

To our knowledge, ours is the first report of using IMRT-SIB with dose escalation resulting in high pCR in adenocarcinoma of esophagus. However, our study has limitations. First, this was a retrospective review that mostly consisted of the patients who were treated by a dedicated multidisciplinary team at a single institution. Selection biases from the study existed. Second, the sample size was small and only ypN was a positive predictive factor in univarate analysis; a larger size study will provide more reliable conclusions. Moreover, although we have reviewed all esophageal adenocarcinoma cases in our institute from the past 10 years, the IMRT-SIB has been implemented only recently and, as a result, the follow-up for this subgroup of patients was short. In a prospective setting, the patients could be stratified by different prognostic clinical variables in an effort to better elucidate the role of SIB dose escalation in certain patient groups.

In conclusion, the dose escalation through IMRT-SIB in the chemoradiation regimen seems responsible for the down staging of the distal esophageal or gastroesophageal junction tumors. The protocol is well-tolerated, postoperative complications were acceptable, and the complete resection rate is high. This radiation therapy dose escala–tion strategy warrants further investigation.

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COMMENTS

Background

Improvement in resection rate is very important for advanced esophageal cancer, balance between toxicity and benefit from neoadjuvant treatment is always the focus of multidisciplinary oncology team. Neadjuvent concurrent chemoradiation is becoming a standard of care for locally advanced esophageal cancer.

Research frontiers

The authors reported here a protocol that improved resection rate through simultaneous integrated boost (SIB) based dose escalation technique without comprising the toxicities. Few reports using this protocol.

Applications

Intensity modulated radiation therapy with SIB (IMRT-SIB) has much safe toxicity profile and less equipment restrain. This finding needs further large phase Ⅲ clinical studies to confirm.

Terminology

IMRT-SIB is a novo radiation technique to deliver much higher dose to the biological gross tumor volume defined by positron emission tomographic positive area without increase radiation dose to all other area surrounding to it.

Peer-review

The authors present a retrospective analysis of an intensified regimen in neoadjuvant chemoirradiation of advanced distal adenocarcinoma of the esophagus. This work is of interest for the oncology community.

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